Molecular Analysis of the Bacterial Community in Table Eggs

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ABSTRACT

The composition of the bacterial flora on surfaces of table eggs is an important factor in influencing the incidence of egg spoilage. Previous studies have focused on a culturing approach for determining bacterial contamination of table eggs. The main problem, however, is culture-based techniques may not adequately describe the bacterial diversity of eggs, since many type of organisms are not cultivated by this method.

This study describes bacterial diversity of table eggs by using both culture-based and molecular approaches. The results of culture based techniques suggested that majority of eggs tested were contaminated with *Staphylococcus* species. No evidence was found for the presence of *Salmonella, Escherichia coli, Campylobacter* or *Listeria monocytogenes*, but *Clostridium perfringens* was found to be positive from 3 eggshells out of 16 shells tested. Methods for direct extraction of bacterial DNA from eggshell and egg content were developed. Cloning of PCR amplified rRNA resulted in the isolation of 91 clones which matched existing sequences in the GenBank database. Eighty-nine % of the isolates were matched to clones of the assigned phylotypes of *Psychrobacter*, Acinetobacter, Staphylococcus, *Clostridium*, *Lactobacillus, Actinobacterium, Proteobacterium, Prevotella, Olsenella* and *Ralstonia*. In addition *Psychrobacter faecalis* and *Psychrobacter maritimus* were isolated from eggshell on TSA at 4 °C, and the characteristics of these bacteria were studied. Interestingly, these bacteria have not been isolated from table eggs in previous studies, and they could potentially be responsible for egg spoilage particularly when the egg are stored in the fridge.

The results obtained in this study will provide valuable information to the egg producers and consumers that may aid improvement of the quality of table eggs and their shelf life. More importantly, it may facilitate the control of spreading these bacteria to the food chain, in order to prevent any food outbreaks that may result from consuming contaminated eggs.

DEDICATION

This thesis is dedicated to my son Eyad, my daughter Lamar, my beloved wife Khadijah, and my parents for their endless loves, supports and encouragements.

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Presentations:

1- 2nd PhD conference, School of Life Science, 2015, Heriot-Watt University, Edinburgh, (UK), Determination of bacterial contamination of table eggs from different housing systems.

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2- 9th Saudi Student Conference, 2016, Birmingham, (UK), Studying microbial diversity of table eggs using part of the 16S rRNA gene.

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1. 1st PhD Conference, School of Life Science, 2013, Edinburgh (UK)
2. 10th Annual Postgraduate Research Conference, HWU, 2015, Eudinburgh (UK)
3. 2nd PhD Conference, School of Life Science, 2015, Edinburgh (UK)

Prize:

1. Prize awarded for the best poster in 1st PhD Conference, 2013.

LIST OF ABBREVIATION

%	percentage
~	approximately
<	less than
>	greater than
≤	less than or equal to
≥	greater than or equal to
16S rDNA	16S ribosomal deoxyribonucleic acid
ANOVA	analysis of variance
API	Analytical Profile Index
BGA	Brilliant Green Agar
bp	base pair
BPA	Baird Parker Agar
$CaCO_3$	calcium carbonate
CCDA	*Campylobacter* blood-free selective agar
CCE	calcium carbonate extraction
CE	caged eggs
CE	Chelex-100 extraction
CFU	colony forming units
cm	centimetre
CTAB	Cetyltrimethylammonium bromide
ddH_2O	Double-distilled water
DEFRA	Department for Environment, Food and Rural Affairs
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside 5´-triphosphate
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
EFSA	European Food Safety Authority
EMBA	Eosin Methylene Blue Agar
EQAPs	egg quality assurance programs
et al.	and others
EtBr	ethidium bromide
EtOH	ethanol
FRE	free range eggs
g	gram
G+C	guanine and cytosine content
GPK	GentraPuregene®kit

h	hours
H_2O_2	hydrogen peroxide
HACCP	Hazard Analysis Critical Control Points
HPA	hybridisation protection assay
I	intermediate
KCl	potassium chloride
KH_2PO_4	monopotassium phosphate
LB	Luria Bertani
LSA	Listeria Selective Agar
MBE	modified boiling extraction
MCS	multiple cloning site
min	Minute
mM	millimolar
Na_2HPO_4	disodium phosphate
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
ND	non-determined
ng	nano grams
OD	optical density
^oC	degree celsius
ORFRE	organic free range eggs
PBS	phosphate buffered saline
PCA	Plate count Agar
PCE	phenol-chloroform extraction
PCR	polymerase chain reaction
pH	a measure of the acidity or alkalinity of a solution
PHE	Public Health England
pmol	picomol
R	resistant
RCA	Reinforced Clostridial Agar
rpm	rotations per minute
S	sensitive
SDS	sodium dodecyl sulfate
sec	second
spp.	species of the given genus
T_a	annealing temperature
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*

TE	Tris-EDTA
T_m	melting temperature
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
TVCs	Total viable counts
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra-violet
VBNC	Viable and but non-culturable bacteria
w/v	weight per volume
µL	micro litre
µm	micro meter

CHAPTER 1 : General introduction

1.1 General introduction

Eggshell and egg content quality are the most critical aspect facing the egg producers in the market. Spoilage of eggs currently cost the industry many of million dollars per year (Roberts, 2004). Therefore, it is of great importance to study the microflora of eggs that affect the quality of eggshell and egg content. Also, it is beneficial to develop a database of bacteria that are associated with eggs to reduce the prevalence and disease potential of these organisms. The main cause of egg spoilage occurs from the growth of Gram negative bacteria within the egg content (Board and Tranter, 1995). Bacteria isolated from table eggs have been investigated in several studies (Alvarez-Fernández *et al.*, 2012; De Reu *et al*., 2008; Adesiyun *et al*., 2005), but little attention has been paid to assessing the microbial diversity of table eggs using the 16S rRNA gene as an identification tool. A study conducted by Adesiyun *et al*., (2005) showed that among 184 tested egg content samples 13 %, 37% and 1.1% were positive for *Salmonella*, *Escherichia coli* and *Campylobacter* respectively, but were negative for *Listeria* spp. Another study by De Reu *et al*. (2008) revealed that natural eggshell contamination of table eggs was dominated by *Staphylococcus* and other Gram-positive bacteria. In another report by De Reu *et al*. (2005), egg content was found to be dominated by Gram-negative bacteria including *E.coli*, *Salmonella* and *Alcaligenes* sp.

Many reports have described how bacterial diversity can be affected by environmental conditions including temperature, soil structure and climate changes (Torsvik and Ovreas 2002; Torsvik *et al*., 1996; Lozupone and Knight 2007). Countries that have high temperature climates such as Saudi Arabia tend to have a different microbial diversity in the environment compared to countries with cold weather (Bahobail *et al*., 2012). They found that table eggs in Saudi Arabia were contaminated with *Campylobacter* and *Listeria*. With increased public interest in natural foods that are free of antibacterial residues or pesticides, food industries have started to produce organic products that are free of chemicals. For example, egg farmers started to produce organic free range eggs, laid by hens which are claimed to be fed organic crops that are neither genetically modified, nor contaminated with pesticides. Also, organic farms cannot use antibiotics for growth enhancement, and the hens have access to the outdoors and are not raised in cages or confined in houses. However, despite the efforts achieved by the egg suppliers to produce organic eggs, there is still an issue that needs to be investigated since eggs laid

in the outdoor environment are likely to be more prone to environmental contaminants from the soil than those laid on clean surfaces (Cox and Cason, 2000). Another critical issue is that the soil contains uncultured bacteria that are reservoirs of new antibiotic resistance genes (Riesenfeld *et al*., 2004). This means if the eggs laid in this environment are contaminated with these bacteria, it may result in transfer of antibiotic resistant bacteria to the human body and cause adverse health consequences.

There has been growing concern about the rise in incidence of food poisoning worldwide. It has been challenging for some countries to detect contaminated food products using available practices (Hu *et al*., 2016). Bacterial outbreaks and food poisoning issues have also occurred in the last decade due to consumption of raw and undercooked food. An outbreak can be defined as two or more confirmed cases involving a particular pathogenic microbe after consuming a food product (Rocourt *et al*., 2003). *Salmonella* outbreaks are commonly associated with eggs and poultry, or products containing eggs as one of their ingredients, such as ice cream and egg mayonnaise. However, there have been substantial increases in the incidence of foods infected with pathogens during the last two decades, in countries that have reporting systems. For example, in 1994, an outbreak of *Salmonella enteritidis* linked to ice cream prepared from eggs infected as many as 224,000 people in the USA (Hennessy *et al*., 1996). Moreover, in the USA, the annual number of food borne disease cases is estimated to be 76 million illnesses, 325,000 hospitalisations and 5,000 deaths (Rocourt *et al*., 2003). The number of illnesses caused by consuming eggs contaminated with *Salmonella* is about 142,000 every year in the USA (FDA, 2015).

In Europe, the European Food Safety Authority (EFSA) stated that in 2013 the total number of salmonellosis cases reported from all the EU countries in humans was 85,268, with a high percentage of the reported cases (44.9 %) due to consuming contaminated eggs (Figure 1.1) (EFSA, 2015). The figure shows that 17 countries reported *Salmonella* outbreaks, with high numbers of occurrence in Poland (114), compared to the UK (9). Also, a report by Public Health England, showed that the total number of cases infected with *Salmonella* from eggs reached 247 cases in 2014, with 158 cases from a single egg source on 15 August 2014 (PHE, 2014). The additional cases are not new infections, but historical cases informed to PHE during that week.

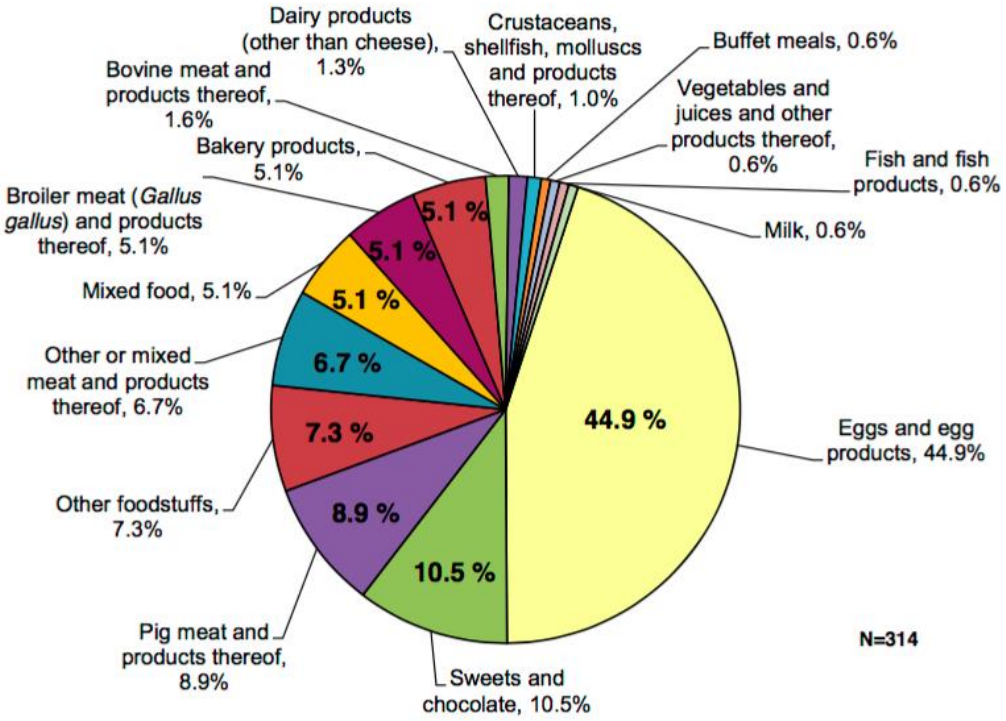

Figure 1.1 Distribution of food products responsible for *Salmonella* outbreaks in the EU in 2013 (EFSA, 2015). Data from 314 outbreaks are included: Austria (7), Belgium (1), Croatia (2), Denmark (4), Estonia (1), Finland (1), France (68), Germany (12), Hungary (5), Latvia (1), Lithuania (6), Poland (114), Romania (4), Slovakia (2), Spain (76), Sweden (1) and United Kingdom (9). Water-borne outbreaks excluded. Other foodstuffs (N=23) include: canned food products (1), cheese (1), herbs and spices (1), and other foods (20). Other or mixed meat and products thereof (N=21) include: turkey meat and products thereof (1), other or mixed red meat and products.

Epidemiological investigators point out that poultry, eggs and meat are the major source of food borne disease caused by bacteria (Luber, 2009), while *Salmonella* and *Campylobacter* are the most frequent bacteria causing food poisoning in humans worldwide (Chemaly and Salvat, 2011). In the late 1980s, eggshell was classified as one of the most common sources of *Salmonella* that can cause salmonellosis in humans (Greig and Ravel, 2009). A report by Okamura *et al*. (2007) described how the incidence of *Salmonella* dropped significantly after introducing a vaccination programme that was applied by the food safety organisations. Despite the improvement, it is still necessary to have effective means of monitoring table eggs for contamination by *Salmonella.*

Viable but non-culturable bacteria (VBNC) are another critical issue that worries both food safety organisations and food producers. Bacteria in a VBNC state are alive but not able to grow on normal laboratory growth media; however, they are still capable of renewing their metabolic activity (Oliver, 2005). Studying VBNC bacteria in eggs can help to describe the microbial diversity of table eggs by using both culture and non-culture based approaches, as well as providing more information about the new emerging micro-organisms. Previous research has focused on identifying bacteria that cause egg spoilage, and penetration of *Salmonella* through the eggshell. However, until now there is no research that has investigated VBNC bacteria using a molecular approach. A metagenomics approach for identifying bacterial isolates can provide information that may assist researchers in estimating the hazard of bacterial isolates, as well as tracing the source of bacterial contamination.

1.2 Food-borne diseases and routes of infections

Food-born diseases are defined as diseases of toxic nature or infections caused by the consumption of contaminated food or water (Le Loir and Gautier, 2003). The infections are caused by many different pathogens, including *Salmonella, Campylobacter, E. coli*, and *Staphylococcus aureus*. The severity of disease caused by bacteria from eggs varies depending on the pathogen and the route of infection; for example, consuming food contaminated with *Salmonella enteritidis* can cause abdominal pain, vomiting and diarrhoea (Barbara *et al*., 2000). Also, complications of *Salmonella* may occur and lead to death among young children or elderly people who are suffering from chronic diseases (Mead *et al*., 1999).

1.3 Economic impact of egg loses

It is estimated by Food and Agriculture Organisation (2011) that the world loses approximately a third of the food produced for human consumption. A recent study by FAO reported that one-third of all food produced is wasted or lost, for which the amount estimated was 1.2 billion metric tonnes annually (Venkat, 2011). Bad egg quality due to bacterial contamination costs the food industry many millions of dollars every year (Roberts, 2004). Recently, 60,000 eggs were destroyed in England after suspicions of contamination with *Salmonella* (BBC, 2015). Also, another report stated that 700,000

contaminated eggs have been recalled and destroyed in the UK (Venkat, 2011). This of course has an impact on the economy, since production of the eggs costs the producers money and energy. Therefore, a fundamental question on food losses would be raised, i.e. whether identifying bacteria associated with food spoilage will decrease the food losses. Also, are the identification methods used for detecting food pathogens efficient and reliable to provide assurance of the safety of food. These questions are important to be addressed to assess food safety and food preservation systems, and develop new identification methodology that is reliable and cost efficient.

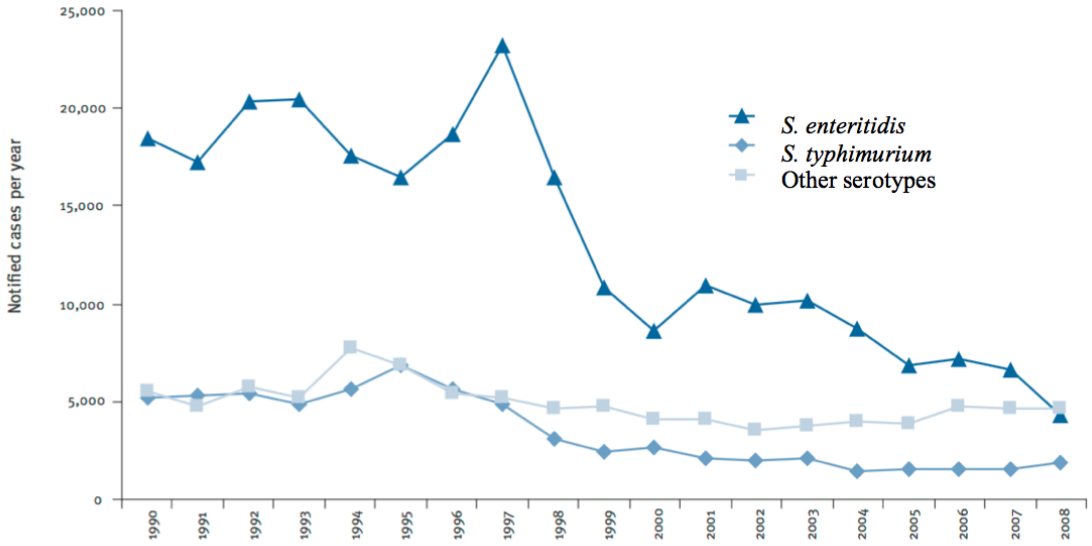

Figure 1.2 Reported *Salmonella* infections in England and Wales, 1990-2008 (Aiken *et al*., 2010).

Effective policies and regulations can address the problem of diseases resulting from food consumption. For example, *Salmonella* was believed to be the main cause of food borne disease in England and Wales until the late 1990s, then the rate of infection has fallen dramatically. Moreover, Foley *et al*. (2011) stated that over the last decades, there has been a significant decrease in the predominant *Salmonella* serovars associated with poultry infections. In a more recent report on *Salmonella* infections, the PHE recorded 293 infected cases in April 2017 (PHE, 2017). The falling trend shown in Figure 1.2 has been attributed to many reasons including public awareness, strict regulations, and more

importantly the compulsory vaccination of the layer hens against *Salmonella* that was introduced in the UK in 1998 (Gantois *et al*., 2006)

1.4 Consumption of eggs

Table eggs are among the most popular and cheap sources of food protein consumed by people worldwide (Papadopoulou *et al*., 1997; Samiullah *et al*., 2013) The European Union is the second largest producer of hen's eggs, after China, with about 6.5 million tonnes estimated production and an average consumption of 235 eggs per capita (Wells, 2010; Alvarez-Fernández *et al*., 2012). In 2014, an average number of 32 million eggs per day were consumed by people in the UK. The production of eggs during the third quarter of 2015 in the UK reached 25,000 tonnes, which was a 1.8 % increase on the third quarter in 2014 (DEFRA, 2015). The report attributes the rise in consumption of eggs to concerns over increasing protein in the diet.

The quality of both eggshell and egg content in terms of microbial contamination can affect the quality of egg products. It has been confirmed that *Salmonella* can migrate from the eggshell to the egg content under certain conditions, (Humphrey *et al*., 1989; Gantois *et al*., 2009; De Reu *et al.*, 2009). Thus, the presence of pathogens in eggs or egg products poses a threat to the consumer either directly through eating a contaminated egg or indirectly through cross-contamination during the handling process. Since eggs are components of many food products, and they are consumed widely, it is essential to ensure the safety of eggs. Egg safety can be implemented by regular testing of eggs for food pathogens and other contaminants. Therefore, developing a rapid, and cost effective method for determining bacteria in eggs is critical in monitoring food pathogens.

1.5 Hen's egg formation and composition

A hen's egg comprises all the raw materials for the formation of the embryo apart from oxygen, which can enter through tiny pores in the shell. If these pores are experimentally clogged, no growth or development will occur (Bellairs and Osmond, 2014). Figure 1.3 shows the structure of a hen's egg. The yolk is about 2-3 cm in diameter and is surrounded by a thin transparent membrane called the Vitelline membrane. The main components of the yolk are proteins and lipids. The egg white (albumen) is enclosed by two shell membranes and is located around the yolk (Board and Fuller, 1994).

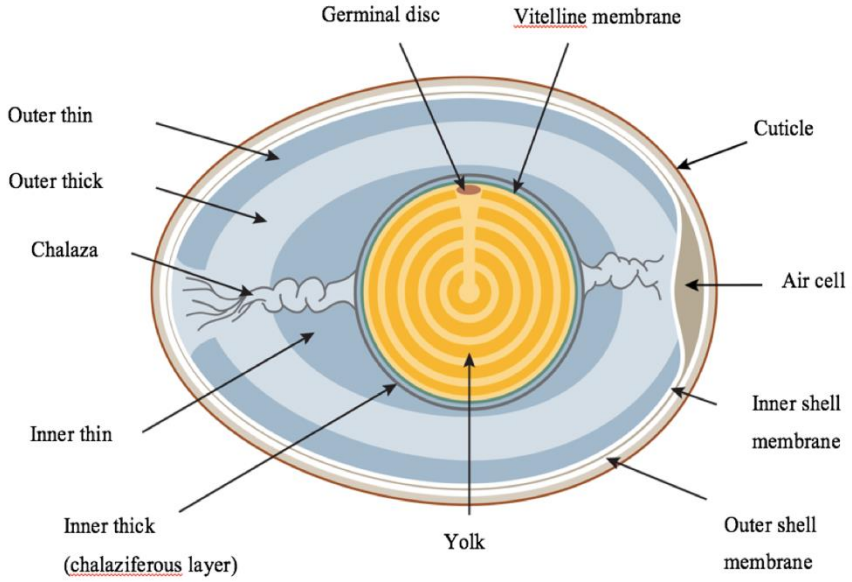

Figure 1.3 Cross section of the egg anatomy showing all the egg's membranes and layers (EBO, 2016)

The eggshell has approximately 8,000 small pores, which allow gas exchange to occur. Inner and outer shell membranes surround the egg white and act as defensive agents against bacterial invasion; the chalazae are balancers that support the yolk and they are intertwined in the opposite direction in order to position the yolk in the center; air cell forms an air space when the egg content cools and contracts, after being laid; cuticle is an outer coat of the shell, also called the bloom, and it acts as a barrier to prevent bacteria from entering the egg; the chalaziferous layer is a dense, fibrous capsule of albumen surrounding the vitelline membrane; the germinal disk is a small circular spot which lies on the surface of the yolk, and gradually supplies the yolk with blood vessels in order to use it for nutrition.

The avian eggshell is comprised of calcite, which mostly contains calcium carbonate ($CaCO_3$), phosphate, chloride, magnesium, and traces of other elements (Nys *et al.*, 2004). It is generally considered that the eggshell is divided into two main portions: the

calcified and the organic fractions, all of which consist of six membrane layers (Perez-Huerta and Dauphin, 2015). The organic part comprises mammillary cores, the shell matrix, the cuticle and the shell membranes. However, these form only a small part of the whole eggshell. Also, the organic membranes contain fibres which constitute a network that envelopes the albumen (Parsons, 1982). The calcified fraction consists of the mammillary knob layer, the outer surface crystal layer and the palisade layer.

The formation of an egg occurs through a complex series of biological processes including ovulation of the yolk from the left ovary into the left oviduct (Figure 1.4). The yolk is collected by the infundibulum where the perivitelline membrane is developed after it is in place for about 15 minutes. The perivitelline membrane forms a sac strong enough to hold the intact embryo and the albumen in eggs. The egg then passes to the magnum and remains for about 3 hours while the egg white is formed. The protein components of the egg white contain antibacterial agents that provide a protection to the yolk (Solomon, 1997). While the egg moves through the magnum, it rotates spirally, and the chalazae and chalaziferous layer are formed. The main function of these structures is to position the yolk in the center of the entire formed egg, in order to protect the nutrient rich yolk from being in contact with external contaminants (Robinson *et al*., 2003). The egg then passes into the isthmus where the two shell membranes are formed, and other minerals and water are added (Solomon, 1991). This stage takes an hour, then the egg transfers to the shell gland or uterus, where it remains for the greatest amount of time, more than 20 hours. At this stage both the albumen layers and the eggshell have been formed.

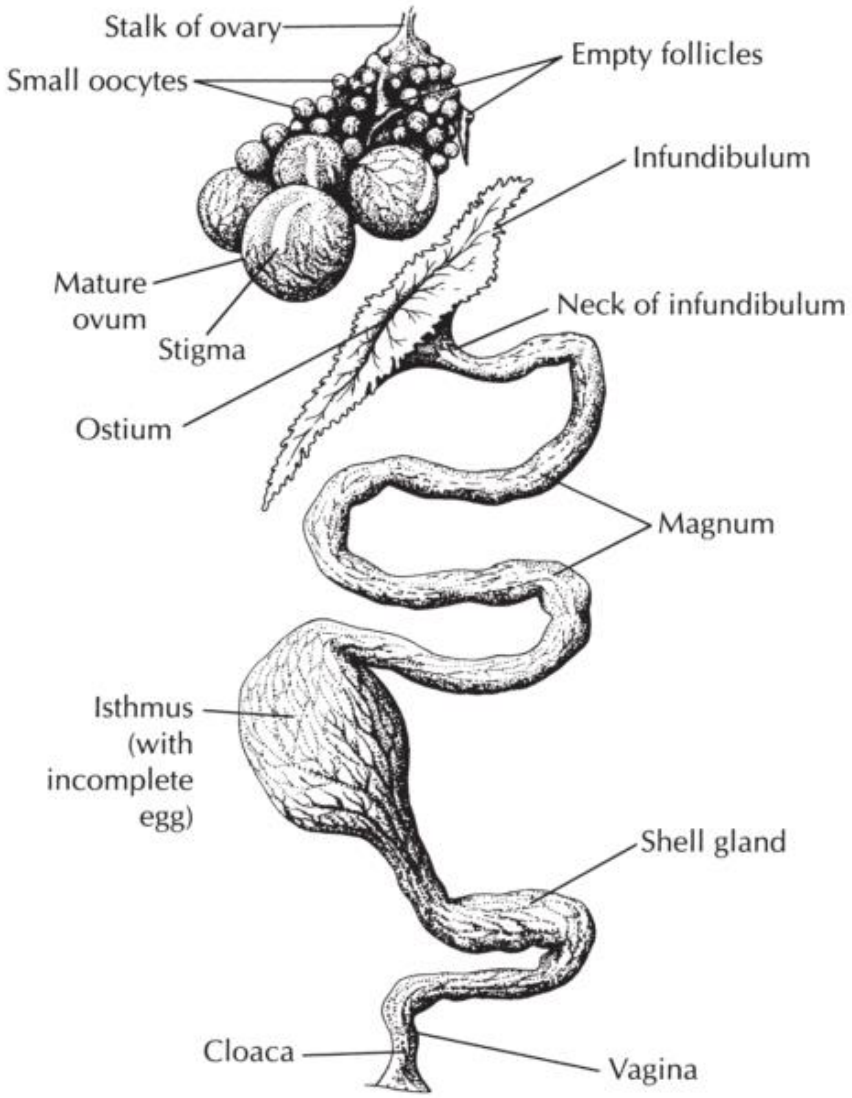

Figure 1.4 Hen's reproductive tract that shows the anatomy of ovary and left oviduct (Bellairs and Osmond, 2005)

1.6 Antimicrobial defences

One of the critical threats to the survival of hen's eggs is the risk of infection by bacteria. Embryos are usually exposed to environmental contaminants that may reduce their survival. The hard shell in the egg, including the inner and outer shell membranes constitutes a physical resistance against bacteria (Mayes and Takeballi, 1983). The cuticle, which is the outside coat of the shell, has a wall thickness of 0.01 mm, and it prevents bacteria from contaminating the egg yolk (Figure 1.5). The egg shell contains between 7,000-17,000 pores. The majority of the eggshell pores are positioned around the equator or the blunt end of the shell. The pore diameter ranges between 9-35 μm (Musgrove, 2004). These pores tend to be wider from the top and become narrower at the bottom, and some of these pores are malformed in their shapes.

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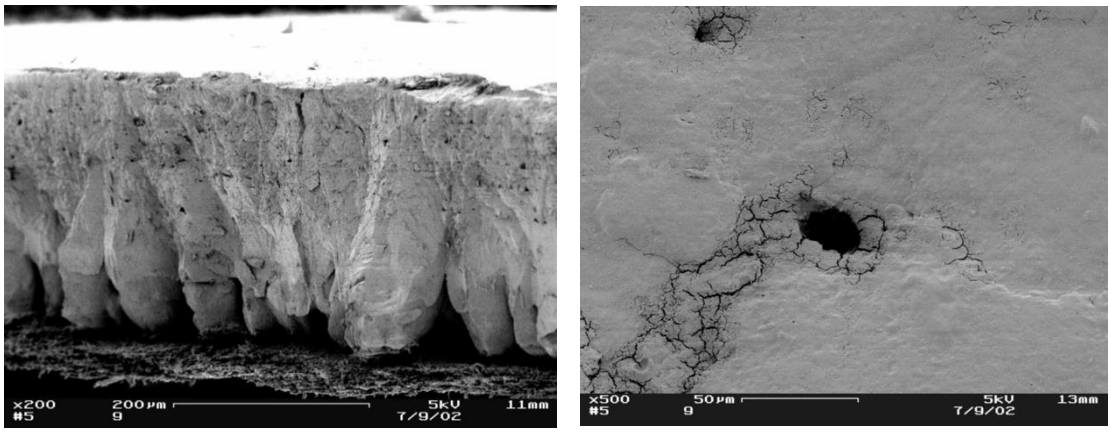

Figure 1.5 Scanning electron micrograph of (A) a cross-section of eggshell, membranes and pores; (B) eggshell showing pores (Musgrove, 2011).

The defense mechanism of eggs against bacteria has been previously described by Board (1982) in which the albumen has two antimicrobial functions for protecting the egg, mechanical and chemical actions. In terms of the mechanical defense, two essential properties are involved in the protection. The viscosity of the albumen impedes micro-organisms from moving towards the yolk. In addition, the second mechanism is the combined function of the chalazae and albuminous sac that contribute to localizing the yolk in the centre of the egg, therefore keeping it far from the shell membranes and the

eggshell which usually contain more contaminants (Board and Tranter, 1995). With regards to chemical properties, egg white proteins constitute the second line of defense against bacteria, since the albumen has a wide range of antimicrobial properties (Table 1.1)

Table 1.1 Characteristics of the main antimicrobial proteins in egg albumen

Protein	Amount of albumen (%)	Characteristics
Lysozyme	3.4	Damages the bacterial cell walls by catalysing hydrolysis of β (1-4) glycosidic bonds in peptidoglycan.
Ovomucoid	11	Acts as a trypsin inhibitor
Ovotransferrin	12	Acts as a chelating agent particularly for metal ions Fe^{3+}
Ovoinhibitor	1.4	Acts as inhibitor of several proteases
Ovoflavoprotein	0.8	Chelates riboflavin, rendering it unavailable for bacteria that require it
Avidin	0.05	Acts as a biotin chelator, rendering it unavailable for bacteria that require it

(Modified from Board and Fuller, 1994)

The biological functions of the albumen act as a hurdle effect that prevents penetration of micro-organisms into the yolk (Naidu, 2000). Furthermore, each one of the natural antimicrobial proteins has a function in protecting the egg; for example, lysozyme functions to hydrolyse the peptidoglycan in the cell wall of bacteria (Board and Fuller, 1994). Lysozyme is also present in the shell membrane and in the cuticle of the calcified shell (Hincke *et al.*, 2000). Another antimicrobial protein, ovotransferrin, is believed to be the cardinal factor for fighting bacteria in eggs (Mine *et al*., 2011). This protein has been identified in the calcified eggshell membrane. It acts as a bacteriostatic filter, which inhibits growth of bacteria in egg white. Moreover, the antimicrobial mechanisms of egg albumen proteins also include bacterial cell lysis, vitamin binding, and metal binding (Stadelman *et al*., 1995).

1.7 Route of microbial contamination of intact eggs

Pathogenic bacteria can contaminate table eggs through two main routes, vertical and horizontal transmission. Vertical transmission of bacteria occurs when the egg contents are contaminated with bacteria during the formation of eggs, a process called transovarian infection. This type of contamination occurs before the shell is formed when the hen is infected with *Salmonella* and particularly *Salmonella enteritidis* which is the most potential human pathogen in eggs (Louis *et al.*, 1988). On the other hand, horizontal transmission happens when the bacteria are transmitted through the eggshell and contaminate the egg content, before or after the egg is laid. Some researchers claimed that contamination of the egg content may occur as a result of initial bacterial deposition on the eggshell surface (Haines, 1938). Moreover, Gentry and Quarles (1972) and Messens *et al*. (2006) suggested that increasing total viable counts (TVCs) of bacteria on the eggshell could consequently increase TVCs in the egg content.

1.8 Extrinsic factors affecting the egg content contamination

1.8.1 Temperature

Temperature is one of the main factors affecting the TVCs of bacteria in any food sample (Scott, 1957). Also, low temperature (chilling, and freezing) is one of the significant factors used to increase the shelf life of perishable food such as eggs. However, storage at low temperature does not prevent growth of all bacteria (Buncic, 2006). In terms of temperature effects on table eggs, a study by Messens *et al*. (2005) showed that temperature enhanced the rate of appearance of red spots on the agar within the eggs, which represents faster growth of *Salmonella enteritidis* on the agar at high temperature. There are many types of bacteria that can survive and grow on food at low temperature including *Campylobacter jejuni*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Aeromonas hydrophila*, *Pseudomonas* and *Psychrobacter* spp. (Venkat, 2011). *C. jejuni* has been previously isolated from the surface of 2 of 226 eggs (Doyle, 1984). *Listeria monocytogenes*, *Yersinia enterocolitica* and *Pseudomonas* were isolated from eggs (Foegeding and Leasor, 1990; Favier and de Guzman, 2005; Ayres *et al.*, 1966). However, *Aeromonas hydrophila* and *Psychrobacter* have not yet been isolated from table eggs.

Pseudomonas was found to be responsible for egg spoilage causing rots in eggs and it can survive and grow at low temperature 4 °C (Haines, 1938).

It is interesting to determine whether the ambient temperature in different countries has an effect on TVCs of bacteria; for example, eggs produced in a low temperature environment may tend to have more psychrophilic organisms.

1.8.2 Moisture

Moisture is required by bacteria to enhance penetration of eggshell and contaminate egg content (Bruce and Drysdale 1994). The penetration can occur in the presence of moisture, and this may occur when eggs allow oxygen to enter the yolk (De Reu *et al*., 2006). According to Padron (1990) the presence of water on the egg shell enhances *Salmonella typhimurium* penetration, but it is not essential for penetration of other bacteria. However, eggs may become moistened when they are removed from the refrigerator temperature to the room temperature and become prone for bacterial penetration in which bacteria will be able to transfer from the eggshell and contaminate egg content. Table eggs, like other food products, contain sufficient nutrients to enhance growth of bacteria. However, there are other several factors that can also encourage, limit or prevent growth of bacteria in table eggs, such as water activity and pH.

1.8.3 Presence of faeces and other contaminants on the eggshell

It is agreed that eggs laid in a heavily contaminated area suffer from bacterial spoilage more that those laid in a clean or less contaminated area (Bruce and Drysdale, 1994). Therefore, the level of egg contamination might be affected by the housing systems, methods of egg handling and equipment hygiene. For example, hens in the free range system may lay their eggs in the environment, therefore, it is likely that eggs laid in the environment contain a higher level of contamination than those laid in cages. The level of eggshell TVCs varies according to the housing system and bacterial isolation methods. Several studies showed the level of contamination ranges from 10^2 to 10^7 CFU/eggshell (Haines, 1938; Alvarez-Fernández *et al*., 2012). Other researchers have focused on the influence of housing system on the level of bacterial contamination (Mallet *et al.*, 2006; De Reu *et al.*, 2008). However, the presence of dirt and faecal contaminants on the eggshell was found to increase the egg spoilage (Svobodova and Tumova, 2014). A study

by Quarles and Bressler (1970) reported that barn housing had on average 9 times more bacteria in the air, and 20-30 times more bacteria on the eggshell than caged housing. Another report by Harry (1963) showed that the eggshells from barn systems had 15 times more bacteria, particularly spoilage organisms, than eggshells from caged systems.

1.9 Table egg housing systems

Egg production regulations have undergone certain changes during the last decade in the European Union (EU). The changes lie in the barren environment and restricted area available in conventional cages and in the welfare of hens. The conventional cage housing system for laying hens was prohibited starting from 2012 in the EU following Council Directive 1999/74/E (Svobodova and Tumova, 2014). From 2012, the only organic free range, free range and furnished caging systems are allowed in the EU. Furthermore, the ban of using the conventional caged system may offer production of better quality eggs in terms of level of bacterial contamination.

In terms of the free range system, according to the EU legislation hens must have access to an outside area through openings called popholes. The advantage of this system is that hens can have access to open run, therefore; healthy hens and eggs may be obtained. A study by Rodenburg *et al*., (2008) to assess hen welfare in enriched cage and non-cage systems showed that the birds in non-cage systems were more active, had stronger bones, were less fearful and made better use of resources (perches, scratching area) than the birds in a caged system. On the other hand, birds in the cage system had a lower mortality rate, weaker bones and were less active. However, hens that have access to outdoors are likely to lay their eggs in the outside environment, and therefore the level of contamination in their eggs may become higher than those laid on a clean surface. Nevertheless, it has been found that eggs from non-cage system had higher microbial load with 1 log unit more contamination of aerobic bacteria than eggs from caged systems (De Reu *et al.*, 2008).

With regards to the enriched cage system, hens are kept in a closed space containing litter, and can move around within the limited area. However, the cage properties of the enriched system differ from the banned conventional non-enriched cage system in several ways, such as scratch mat area, a nest box area, perches and more importantly space per bird (Batt and Robinson, 2014). The enriched caged system has more space than in

conventional non-enriched cages, 750 cm^2 versus 550 cm^2 per bird. Also, hens have more shared space, access to the perch, nest and litter area for scratching and pecking (Sandilands and Hocking, 2012). Therefore, reduction in the level of contamination in eggs may be expected as long as the hens are not suffering from microbial diseases.

The level of contamination in table eggs from different housing systems has been determined by researchers from different countries, but no studies have estimated bacterial contamination of table eggs in Scotland. It is likely that some extrinsic factors including temperature and water activity might indirectly affect the TVCs of eggs. The level of eggshell contamination can be very high, 10^2 to 10^7 CFU/eggshell for grade A eggs collected from local groceries in different countries. Also, current European Union legislation prohibits washing of grade A eggs (Hutchison *et al*., 2003). However, it is believed that washing the eggs can reduce the level of eggshell contamination to half of the initial microbial load (Musgrove, 2011). This regulation in the EU is in contrast to that elsewhere in the world, with countries such as Saudi Arabia, Japan, USA and Australia allowing the washing of table eggs. Thus, variation in the level of eggshell contamination estimated in different countries should be taken into account.

1.10 The microbial community in table eggs

Table eggs contain several intrinsic parameters that can protect the egg yolk from any bacterial attack. These parameters discussed earlier in this chapter include both the physical shield protection, provided by the eggshell and the presence of natural antimicrobial proteins in the egg white. However, components of the egg yolk are excellent nutrients for microorganisms. Thus, occurrence of numerous pathogens and other organisms on the egg shell may increase the chance of egg shell penetration and transfer of these pathogens to the egg content under appropriate conditions (Samiullah *et al*., 2013; Howard *et al*., 2012; Chousalkar *et al*., 2010; De Reu *et al*., 2006b). The speed at which bacteria can enter and spoil the eggs depends on factors such as temperature, time and storage conditions (Jay, Loessner and Golden, 2008). Furthermore, the level of bacterial contamination is another major factor that can affect the process of spoilage (De Reu *et al*., 2009).

There are several bacterial species that have become a concern to the egg industry and food safety agencies. *Salmonella enteritidis, Campylobacter jejuni and Escherichia coli* have been implicated in egg-borne diseases (Musgrove, 2011). Other organisms found in eggs and considered as spoilage bacteria are *Listeria monocytogenes*, *Proteous melanovogenes*, *Yersinia enterocolitica* and *Pseudomonas* spp. A number of studies have described the bacterial community of table eggs using conventional identification methods. Among the bacterial genera found in eggs are *Aermonas, Alcaligenes, Escherichia, Micrococcus, Salmonella, Acinetobacter, Pseudomonas, Proteus, Flavobacterium* and *Staphylococcus* (Jay *et al*., 2008). Also, most of the previous works have focused on the bacteria isolated from spoiled and rotten eggs (Elliott, 1954; Haines, 1938; Haines and Moran 1940; De Reu *et al*. 2008).

Rotten eggs comprise a complex bacterial community of Gram-negative and few organisms of Gram-positive. Many of the contaminants are members of the genera *Pseudomonas, Aeromonas, Proteus* and Alcaligenes (Mayes and Takeballi, 1983). Table 1.2 shows the frequency of bacterial occurrence isolated from eggshell and spoiled egg content, in which some bacterial genera tend to be found more on eggshell than in egg content. Board and Halls (1973) showed that *Micrococcus* and *Staphylococcus* constituted the major flora of table eggs, while *Enterobacteriaceae* and *Streptococcus* were also important components. Also, De Reu *et al*. (2008) described that the eggshell is dominated by Gram-positive bacteria, whereas Gram-negative bacteria have a greater tendency to overcome the antimicrobial defences of the egg content.

In the above studies, the identification methods used have involved studying the morphological characteristics of the isolates by growing the bacteria in selective media, and identifying the colonies and cell morphologies. Other recent studies have used biochemical tests for identifying bacteria (Adesiyun *et al*., 2005; De Reu *et al*., 2006b; Arathy *et al*., 2009). Nevertheless, molecular identification using the 16S rRNA gene might uncover new bacterial species that have not previously been identified. Despite the availability of the PCR technology and the 16S rRNA gene application, little attention has been given to the importance of a metagenomics approach in studying bacterial diversity of table eggs.

Table 1.2 Types of bacteria isolated from the eggshell and content of a rotten egg.

Genus	On the egg shell	In rotten egg contents
Micrococcus	+	+
Achromobacter	+	+
Aerobacter	+	-
Alcaligenes	+	+
Arthrobacter	+	+
Cytophaga	+	+
Escherichia	+	+
Flavobacterium	+	+
Pseudomonas	+	+
Staphylococcus	+	+
Aeromonas	+	+
Proteus	+	+
Sarcina	+	-
Serratia	+	-
Streptococcus	+	+

[a] Bacterial isolation represented by (+) signs, the signs (-) mean bacteria was not isolated. Source adapted from De Reu *et al*. (2008).

1.10.1 *Salmonella* in table eggs

Salmonellosis is one of the critical issues and *Salmonella* is considered as a significant food-borne pathogen that contaminates food worldwide (Singh *et al.*, 2010). Moreover, it costs the food producer a great economic loss, particularly for poultry and egg producers as a result of high morbidity and mortality of young chickens. The species *Salmonella enteritidis* has been frequently linked to outbreaks associated with foods prepared from table eggs (Morse *et al.*, 1994; Camps *et al.*, 2005; Tu *et al.*, 2008; Dyda *et al.*, 2009).

S. enteritidis is a bacterial strain that can cause gastrointestinal illness and is frequently associated with consumption of eggs or poultry. The symptoms in humans include stomach pain, cramps, vomiting and fever. The poultry intestinal tract is the main reservoir of *Salmonella*, and contamination of eggs could occur when they are being formed. In 1980s, there was a dramatic increase in the number of people infected with *S. enteritidis* in the United Kingdom (UK) (Murchie *et al.*, 2008) Furthermore, in 2014, a number of 247 people were infected by *S. enteritidis* associated with consumption of eggs (PHE, 2014). However, continuous monitoring and testing of table eggs for the presence of *Salmonella* and other pathogens might reduce the prevalence of outbreaks and improve the egg quality which in turn enhances the economy.

1.10.2 *Escherichia coli* in table eggs

E. coli is another problematic bacterium, and is recognised as one of the most common bacteria that can be found in the gastrointestinal tracts of animals and humans. Also, 10% of *E. coli* strains are opportunists and categorised as a faecal indicator (Alvarez-Fernández *et al.*, 2012). *E. coli* has been found to be one of the most common isolates from eggshell (Jones *et al.*, 2015). A study conducted by Chousalkar *et al*. (2010) pointed out that the prevalence of *E. coli* from 500 eggs tested was 45 eggs were positive, and in all cases the bacteria were isolated from the eggshell. Another study by Alvarez-Fernández *et al*. (2012) found that a total of 120 out of 240 egg samples tested were positive for *E.coli*.

1.10.3 *Staphylococcus* in table eggs

Staphylococci are Gram-positive bacteria, that are usually isolated from the environment, animal tissue and human skin (Kloos, Zimmerman and Smith, 1976; Nagase *et al*., 2002). In addition, they are considered as the cause of serious illness that leads to losses in poultry production, including infection of subcutaneous tissue (cellulitis) and local inflammation of the skin (dermatitis) (Cheville *et al*. 1988). The significant increase in staphylococcal infections in poultry flocks may pose a threat to consumers leading to epidemiological diseases (Stepień-Pyśniak *et al*., 2009). Furthermore, the rise in staphylococcal infection in chickens might increase the occurrence of these bacteria in table eggs (Adesiyun *et al*., 2005).

1.10.4 Other bacteria in table eggs

There are other enteric pathogens that have been isolated from eggs or egg products, such as *Campylobacter jejuni*, *Listeria monocytogenes*, and *Clostridium perfringens* (Adesiyun *et al*., 2005; El-Jakee *et al*., 2013). *C. jejuni* is commonly associated with poultry, therefore; it is possible that the eggshell or egg content may become contaminated. A study by Doyle (1984) showed that 2 eggshells of 226 eggs were contaminated with *C. jejuni.* In another study by Sahin *et al*. (2003) a total of 1000 eggs tested and no *C. jejuni* was isolated.

L. monocytogenes was isolated previously from the eggshell in a study by Nitcheva *et al*. (1990), one eggshell was found positive for *L. monocytogenes* from 71 samples tested. *Pseudomonas* was found to be one of the main egg spoilage bacteria (Elliott, 1954). A study by Alvarez-Fernández *et al*. (2012) found that *Pseudomonas* spp. were the second major contaminant of eggshell. *Yersinia enterocolitica* is another bacteria that was isolated from eggs using enrichment culture in which a prevalence of 2.27% eggshells were found positive from 352 eggs tested, but no *Y. enterocolitica* was detected from egg content samples (Favier *et al*., 2005)

1.11 Source of bacterial contamination

Sources of eggshell contamination may include housing materials, soil, water, human and animal skins, feathers, blood, dust, handling elements and faeces (Board and Tranter, 1995). The extent of contamination is relatively affected by the cleanliness of these surfaces (Board and Tranter, 1995). In a study by Harry (1963) examined the relationship of the eggshell bacterial flora to the environment and found that the predominant types of bacteria that can grow at 37 °C were present in the shell and membrane of eggs from battery units and deep litter pens. Also, the TVCs of aerobic bacteria in the shell and membranes of all the deep litter was found to be about 15 times more than of the battery eggs. Moreover, higher incidence of aerobic sporing bacteria, *Pseudomonas* and *Staphylococcus* were found in the deep litter eggs. However, the bacteria of the litter may transfer to the feathers and feet of the laying hens which in turn contaminate the eggshell.

TVCs from eggshell fluctuate widely from 10^2 to 10^7 CFU/eggshell depending on the egg treatment performed before they are sold, and the average TVC is considered to be 10^5 for unwashed eggshell (Board, 1966). Furthermore, eggs carrying visible dirt and faeces, may have TVCs that reach millions of bacterial cells (Mayes and Takeballi, 1983). A study by Moats (1980) has determined percentages of types of microorganisms classified from isolates from five washed and unwashed eggshells (Table 1.3). The result showed high proportion of Gram-positive cocci on the unwashed eggs and included as *S. faecalis and Aerococcus.* Most of the organisms found on the eggshell are harmless from the standpoint of spoilage of shell eggs. Also, the researcher found washing eggshells that are heavily contaminated with bacteria particularly with colder water increases the chance of egg content to become contaminated.

Table 1.3 Percentages of types of microorganisms classified from isolates from five eggs

Microorganisms	Microorganism percentages in:				
				Eggs	
	Washwater (73)[b]	Brushes (93)	Conveyors (77)	Unwashed (93)	Washed (96)
Group1 (gram-positive cocci)					
Total	59	20	52	71	43
Micrococcus	33	19	26	15	11
Staphylococcus					
S. aureus	3	-	4	3	7
Other	23	1	22	38	24
Aerococcus	-	-	-	8	-
Streptococcus faecalis	-	-	-	8	-
Group 2 (gram-positive and gram-variable rods)					
Actinomycetes					
Total	17	50	31	15	40
Arthrobacter	8	23	14	4	12
Kurthia	1	4	4	1	7
Propionibacterium	-	-	-	2	-
Microbacterium	-	-	-	-	1
Other (unidentified)	7	23	13	8	19
Bacillus	-		-	-	2
Lactobacillus	-		-	1	-
Group 3 (gram-negative rods and cocci)					
Alcaligenes	11	12	3	-	4
Moraxella	3	11	9	-	5
Acinetobacter	3	4	3	1	
Flavobacterium	4	2	3	1	1
Acetobacter	3	-	-	-	
Escherichia coli	-	-	-	10	3
Group 4 (yeasts)	-	-	-	1	1

[a] Isolates were from equipment surfaces, washed and unwashed eggs, and washwater. Because of rounding, some totals may not add up exactly; [b] Parentheses indicate total number of isolates classified. Source Moats, 1980.

1.12 Viable but non-cultivable bacteria

There is increasing evidence regarding the presence of microorganisms in a VBNC state in food samples, particularly foods that are exposed to environmental stressors and varied processing treatment strategies (Rowan, 2004). This issue should be considered as a critical point, since the evidence is that bacterial pathogens may recover their ability to cause illness after ingestion by consumers despite their failure to grow under the conditions applied when determining their presence in foods. As can be seen from Figure 1.6, *Vibrio vulnificus* enters into a VBNC state when incubated at 5 °C (Oliver, 2005).

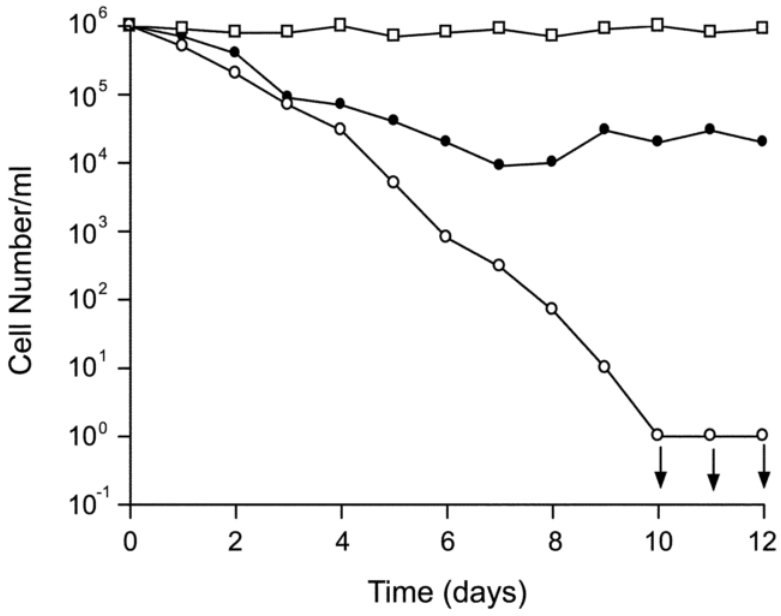

Figure 1.6 Entry of *V. vulnificus* into the VBNC state on incubation at 5 °C. Shown are total cell counts (□), culturable counts (○),viable counts (●) and (↓) shows the days where bacteria fail to grow and enter in to VBNC state. Source Oliver (2005)

The culturable curve declines rapidly when the bacteria are exposed to one or more environmental stresses. However, during this period of decline, TVCs remain constant. The indicator test that determines whether bacterial cells are alive but in a VBNC state or dead, is the viability count. Several methods can be applied such as assays to show this trait, but generally these characterise some aspect of metabolic activity which indicates that the cells are alive. Also, Bacterial cells enter in to the VBNC when they are exposed to natural stress, such as incubation outside the temperature range, starvation, elevated oxygen or osmotic concentration and exposure to light (Oliver, 2000).

The number of bacterial species described to enter in to a VBNC state until now are about 60 species, including large numbers of human pathogens. These pathogens include *Campylobacter* spp., *Francisella tularensis*, *E. coli*, *Helicobacter pylori*, *Listeria monocytogenes*, *Legionella pneumophila*, *Mycobacterium tuberculosis*, several *Salmonella* species, *Pseudomonas aeruginosa*, *Shigella* spp. and *Vibrio cholerae*, *V. vulnificus* and *V. parahaemolyticus* (Oliver, 2005).

1.13 Bacterial cross-contamination

Cross-contamination of materials with food pathogens while processing food at home is considered to be a major factor in epidemic and sporadic foodborne illness (Scott, 1957; Chen *et al*., 2001). During food preparation or handling, bacteria that are deposited on the eggshell can be transferred to processing equipment and surfaces, for instance cutting boards, knives and food appliances (Miller *et al*., 1996; Zhao *et al*., 1998). Thus, proper hygiene including frequent hand washing could decrease the chance of contamination occurring.

A study by Humphrey *et al*. (1994) showed evidence that during preparation of dishes using eggs artificially inoculated with *Salmonella*, a significant cross-contamination of work surfaces, hands and food equipment occurred in the kitchen. In addition, as already described the eggshell carries a wide range of bacterial diversity and some of these organisms may not yet have been identified and their potential for cross-contamination is still therefore unknown.

It is true that the eggshell is not a consumable part, but when handling and preparing food dishes containing eggs as an ingredient, a large number of bacterial cells might transfer and contaminate the working surfaces. For instance, the average surface area of an egg is estimated to be 60 cm^2, and if it is assumed that holding an eggshell for food preparation leads to transfer of all the bacteria on the touched surfaces to the hand, in which each finger tip is estimated to be 2 cm^2, this means about 33% of the eggshell microbial counts may transfer, which is probably enough to cause an illness. Therefore, it is essential to study the bacterial diversity of the eggshell and assess the hazards that can be caused, if bacterial cross contamination occurs. Moreover, some micro-organisms on the eggshell might not cause gastroenteritis or other instant illnesses, but may instead be considered as life threatening pathogens. For example, *Acinetobacter baumannii* has emerged as one of the troublesome bacteria for health care institutions worldwide. It is resistant to all types of antibiotics, and can survive for prolonged periods in the environment (Peleg *et al*., 2008). A study by De Reu *et al*. (2006a) has isolated *A. baumannii* from the eggshell but there was no evidence the presence of *A. baumannii* within egg content.

1.14 Types of egg products involved in outbreaks

The bacteria commonly found in eggs that are known to cause food poisoning, have already been described. There are various types of food prepared from raw eggs or undercooked eggs that can lead to food poisoning, for instance, mayonnaise, ice cream and some types of pasta. An outbreak in 2015, infecting 90 people at Melbourne's Langham Hotel was caused by *Salmonella* from raw egg mayonnaise (AAP, 2015). According to FDA (2004), homemade ice cream caused outbreaks of *Salmonella* infection every year, with 17 outbreaks from 1996 to 2000 resulting in more than 500 cases of illness in the USA, and the ingredient responsible for the outbreaks was undercooked eggs. Food products that contain raw or undercooked eggs are described in Table 1.4.

Table 1.4 Classification of egg containing foods

Partially cooked egg	**Undercooked / Raw egg**
- Homemade meringues	- Fresh Mousse
- Lemon curd	- Mayonnaise
- Quiche / flan / Spanish tortilla	- Some types of ice creams
- Scrambled egg	- Some sorbets
- Boiled egg	- Royal icing
- Fried egg, Egg fried rice	- Horseradish sauce
- Omelette	- Tartare sauce
- Poached egg	- Raw egg in cake mix
- Egg in batter, breadcrumbs e.g. Scotch egg	- Some types of cheeses contain egg protein.
- Homemade products where egg is used to make breadcrumbs to stick to fish/chicken	- Salad cream
- Hollandaise sauce	
- Egg custard, Creme Brulee, Creme caramel	
- Homemade pancakes and some Yorkshire pudding	
- Bread and butter pudding	
- Fresh egg pasta	
- Tempura batter	

Source BAF (2015)

1.15 Interventions to improving microbial quality of eggs

After the spread of *Salmonella* from eggs in the UK in 1978, interventions to reduce the incidence of *Salmonella enteritidis* were introduced. One of the interventions was setting up of egg quality assurance programmes (EQAPs), which are a monitoring practice applied to egg production farms. The programmes involve applying a monitoring tool such as Hazard Analysis Critical Control Point (HACCP) that is designed to assess and detect the critical points in any egg production systems that may lead to contamination (Mumma *et al.*, 2004). A reduction in the presence of *Salmonella enteritidis* in table eggs, and the number of *Salmonellosis* cases resulted after applying the EQAPs (Louis *et al.*, 1988; Hogue *et al.*, 1997). Therefore, applying these monitoring tools can reduce the vulnerability of eggs to contamination, with the aim of providing high quality and safe egg products to consumers.

1.15.1 Egg traceability

The EU has applied a regulation to allow tracing of table eggs. Directive 2002/4/EC states that all class (Grade) A eggs sold in the market within the EU must be stamped with a code that facilitates identifying of where the eggs come from. The label provides information about the country of production and method of production, for instance organic, free range and caged eggs (Figure 1.7).

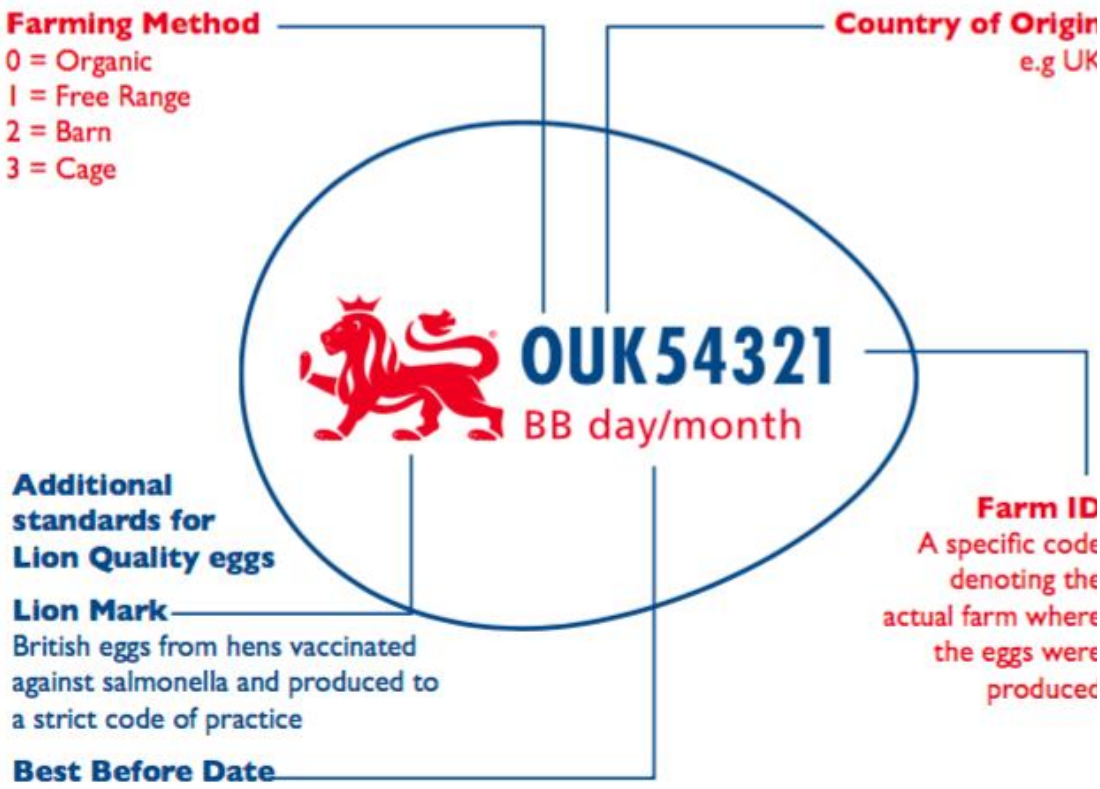

Figure 1.7 Egg traceability system. The eggs are labelled with codes and each code represents a property (BLQ, 2007)

1.16 Bacterial isolation and enumeration

Isolation of bacteria from table eggs has been demonstrated in several studies. For enumerating the TVCs of the eggshell, some studies have used a sterile swab moistened in a buffer to wipe a part of the eggshell surface (Adesiyun *et al*., 2005; Chousalkar *et al*., 2010; Chaemsanit *et al*., 2015). Another method involves placing an intact egg in a sterile plastic bag containing 10 ml diluent buffer (Bahobail *et al*., 2012; Gole *et al*., 2013; Jones and Anderson, 2013). Bacterial isolation using the swab technique represents only the part of the surface area which is smeared, and the result is calculated by using a formula to obtain the estimated TVCs of the whole egg, whereas the plastic bag method should isolate bacteria from the entire eggshell surface. In terms of enumerating the TVCs from the egg content, the standard protocol involves 25 g of the egg content being homogenised with 225 ml of sterile diluent for 60 seconds (Sun, 2011; Batt and Robinson, 2014; Harrigan, 1998).

1.17 Bacterial detection and identification

Public health concerns about food borne pathogens have increased, and there is a rise in public demand to ensure safety of food products sold in the market. Accordingly, continuous development of methods for detection of bacteria must take place, and new rapid detection methods that can accurately provide adequate information about food pathogens is required. Improvements in detection methods have resulted from developments in the field of molecular biology, biochemistry and computer science. Rapid and sensitive identification methods are now available in the form of analytical kits or protocols that can be routinely used in the lab. However, it is true that each method has a limitation, such as the cost, time or sensitivity of the method used, so continuous optimisation of the identification methods is essential. For example, one of the greatest challenges that faces the microbiologist is sample preparation, since an efficient method for detaching bacteria from the food sample is always required.

1.17.1 Conventional methods

The conventional methods used extensively by the microbiologist in the past have relied on appropriate growth media to isolate and enumerate bacterial cells. This approach is inexpensive, sensitive and informative (Doyle and Buchanan, 2012). Moreover, this method can be applied in any microbiology lab. However, it is time consuming since it may involve several steps, including pre-enrichment, selective plating, and studying morphological characteristics (Mandal *et al.*, 2011). Hence, in order to identify the bacteria using conventional methods, several days and considerable efforts are required.

1.17.2 Methods based on biochemical analysis

Biochemical tests are widely used for rapid identification of bacteria, through determining the metabolic properties of the test bacterium, and comparing them with the characteristics of known microorganisms. The tests can involve determination of single enzymes such as catalase, oxidase, urease and coagulase, or the presence of a metabolic pathway detected using assays based on fermentation of different carbohydrates, and the ability to degrade amino acids (Harvey, Champe and Fisher, 2007). However, the limitation of using these tests is variability of the results obtained by different labs (Verma and Singh, 2014).

A majority of the studies conducted previously for identifying bacterial isolates have used biochemical and serological methods (Haines, 1938; Adesiyun *et al*., 2005; Alvarez-Fernández *et al*., 2012; Reu *et al*., 2008). Serological methods are based on techniques initiated from immunology. The concept is that animals that have been injected with bacterial antigens produce antibodies in their blood that identify these particular antigens through binding to them with high affinity. Two types of antibodies can be used for bacterial identification; monoclonal or polyclonal antibodies. Serological methods can be used to detect bacterial genera, species and serotypes. The most commonly used serological tests for identifying bacteria are immunofluorescence assays and enzyme-linked immunosorbent assays. Serological methods can identify target bacteria rapidly, but the accuracy of these methods relies on the specificity of the antibodies used. For example, the use of monoclonal antibodies instead of polyclonal antibodes might improve specificity.

It has been demonstrated that nucleic acid based method are very successful for determining environmental bacteria, since they can provide specific details that can help to study the diversity of organisms in a tested sample.

1.17.3 Molecular identification using part of the 16S rRNA gene

The 16S rRNA gene has been by far the most common applicable gene marker used for many reasons, including presence of this gene in all bacteria, as well as that the function of this gene has not changed over time, which makes the gene a more accurate measure of evolution through time (Janda and Abbott, 2007). In food safety, it is critical to detect and trace any source of microbial contamination, since this information will assist inspectors in treating and eradicating the contamination problem. For example, in 2014 a national outbreak of *Salmonella enteritidis* that affected 247 people in England was linked to consumption of eggs from a single source (PHE, 2014), and this was determined by 16S rRNA gene sequencing.

Determining microbial diversity of environmental or food samples is one of the main applications of 16S rRNA sequence analysis. Nevertheless, a majority of bacterial species in these samples may still be uncultivable in the laboratory, due to the fact that growth requirements are unknown (Rajendhran and Gunasekaran, 2011). Therefore, another important advantage of using 16S rRNA gene is the ability to identify uncultured bacteria that can be present in a food sample, and pose a threat to the consumers.

During the last two decades, bacterial diversity of environments such as soil, ocean surfaces, animal rumen and human skin has been determined by this approach, and many new organisms were identified. The cloning and sequencing of directly amplified 16S rRNA genes from these environmental samples using a metagenomic approach can extensively describe the microbial diversity more completely than using conventional culture-based studies (Handelsman, 2004). In this way, knowledge of bacterial diversity and species distribution has been greatly extended by directly applying 16S rRNA sequence analysis to nucleic acids isolated from environmental or food samples (Felske and Weller, 2004).

1.18 Aim the project

The aim of this project was to study bacterial diversity of table eggs using both culture-bases and molecular approaches. The first part of the project involved estimating the TVCs from both eggshell and egg content. Afterwards, bacteria were selectively isolated on different types of growth media. Then, isolates were selected based on their morphological characteristics and identified by analysis of part of the 16S rRNA gene.

The second part of the project was to develop a methodology for extracting bacterial DNA directly from the mixed bacterial community of both eggshell and egg content. Amplicons were then individually cloned and sequenced to identify cloned isolates.

The cloning approach identified that psychrophilic bacteria were present in eggs. The final part of the project was therefore directed at examination of these bacteria, since they can grow at low temperature and may lead to egg spoilage and decreasing of the shelf life of table eggs.

CHAPTER 2 : Bacterial contamination of table eggs from different housing systems

2.1 Introduction

Table eggs are considered as one of the essential ingredients in many processed foods, some of which are prepared from raw or undercooked eggs. A number of studies of egg contamination and the consequences that might occur as a result of consuming contaminated eggs have been previously published (De Reu, 2006; Messens *et al.*, 2006; Alvarez-Fernández *et al.*, 2012; Gentry and Quarles, 1972). Many factors can affect the microbial load in a food sample including temperature, nutrient requirements and storage condition (Swatland, 2000). Moreover, regulations applied to food production by local authorities may affect the number and type of the organisms present in a food sample. For example, washing of class A table eggs to remove contaminants is not allowed in the European Union. A study by Hutchison *et al.* (2004) found that washed eggs had significantly lower microbial load than unwashed eggs. Therefore, the microbial counts of table eggs may vary from one place to another depending on the parameters already mentioned.

Bacterial communities associated with table eggs have been described using culturing techniques and the conventional identification methods that are based on studying morphological characteristics (colonies and cells) of the isolated bacteria (Adesiyun *et al*., 2005; De Reu *et al*., 2009; Salihu, Garba and Isah, 2015). Other studies have used biochemical tests as an identification tool (Safaei 2011; Chaemsanit *et al*. 2015; Arathy *et al*. 2009; Alvarez-Fernández *et al*. 2012). Evaluating bacterial communities using these methods might provide initial information but may not accurately describe the full range of microbial diversity. However, using methods based on nucleic acid analysis has the potency to provide genus and species identification for each isolate (Janda and Abbott, 2007). Moreover, it can also provide useful information for tracing source of contamination.

The eggshell is an essential structure for two main reasons. Firstly it forms an embryonic chamber for the chick development, which controls gas exchange medium and provides protection to the chick. Secondly, it acts like a shield that protects egg content from contaminants which could spoil it, and therefore decrease the shelf life of table eggs. The eggshell contains 97% calcium carbonate, and this is obtained by the hen in the diet (Arukwe and Goksoyr, 2003). It has been hypothesised that egg content contamination

might occur as a result of bacterial accumulation on the eggshell after the egg has been laid on a contaminated surface (Haines, 1938).

Board and Tranter (1995) reported that the level of contamination of commercial eggs varied between 10^2 to 10^7 CFU/eggshell with an average of about 10^5 CFU/eggshell. There is a weak correlation of the level of contamination and the appearance of the shell, with the exception of heavily soiled shells. Therefore, clean eggs can harbour on their shells more organisms than clearly soiled shells. The available data showed that Gram-positive bacteria dominate the shell flora possibly because of their tolerance to dry weather, and *Streptococcus*, *Staphylococcus* and *Micrococcus* are the major contaminants. In contrast rotten eggs found to be contained a mixed infection of Gram-negative and a few Gram-positive bacteria, and the most common contaminants are the genera *Alcaligenes*, *Acinetobacter*, *Serrtia*, *Pseudomonas*, *Citrobacter*, *Proteus* and *Aeromonas* (Board and Tranter, 1995) .

A study carried out by De Reu *et al*. (2008) compared the level of eggshell contamination from conventional cages with eggs from furnished cages. The TVCs was found between these systems to be 10^4 to 10^5 CFU/eggshell. Also, the study showed a significant difference between caged and non-caged systems in terms of TVCs accumulation on eggshells. Moreover, eggshells from caged systems had slightly but significantly ($p<0.001$) lower contamination than non-caged systems. It is crucial from a food safety perspective to study the bacteria that contaminate and deteriorate table eggs, as well as from an economic perspective to improve their shelf life (Gole *et al*., 2013).

Different methods for isolating the bacteria from the eggshell include washing the entire egg in a sterile bag. The method involves washing the entire eggshell in a sterile plastic bag containing phosphate buffer saline in which the bacteria are extracted in the saline, then a measured sample from the suspension is cultured on media plates for enumeration and bacterial isolation (Gentry and Quarles, 1972). The advantage of using this method is that it allows a wide range of bacterial species to be isolated since the entire eggshell is treated. Another method used for bacterial isolation involves crushing the shell with membranes after evacuating the egg content in a sterile bag containing a diluent (Haines, 1938). This method is appropriate in terms of studying bacterial transfer from the eggshell

to egg content particularly to enumerate and identify types of bacteria that are trapped in eggshell pores.

Swab-sampling is another method that has been used for enumerating bacteria from the eggshell surface (Methner *et al*., 1995). The method includes swabbing part of the eggshell with a swab moistened with PBS and soaked off in 10 ml PBS solution. This method can be used for enumerating bacteria from a sample surface but it is not efficient for studying the bacterial diversity of the entire eggshell.

Most of the previous research that studied bacteria of table eggs were performed before 1990, and mostly focused on egg spoilage and hatching eggs (Mayes and Takeballi, 1983). Few recent studies have determined bacterial contamination of table eggs using conventional methods for detecting bacteria (De Reu *et al.*, 2008; Alvarez-Fernández *et al.*, 2012).

2.2 Objective

The initial objective of this study was to determine the bacterial contamination of table eggs, including both eggshell and egg content. Isolation of bacteria from eggshell rinse and egg content homogenate was performed on plate count agar (PCA), to obtain the TVCs, while samples were plated on different selective media for targeting particular bacteria. Also, DNA isolation and sequencing of part of the 16S rRNA was used as a tool to identify additional bacteria present which may contribute to egg spoilage.

2.3 Materials and methods

2.3.1 Egg sample collection

A total of 88 eggs were collected from different local groceries in Edinburgh. Of these 22 were organic free range eggs, 33 were free range eggs and 33 were caged eggs and collected on different occasions. All eggs were transferred to the lab, in ambient conditions, and examined for microbial quality on the same day as purchasing. A number of 3 eggs from each origin were tested as shown in Appendix I. All sampled eggs were handled using sterile tweezers and under aseptic conditions. A pre-inspection treatment was performed visually, in which eggs that had a crack, blood or faeces contamination were excluded from the experiment.

2.3.2 Buffers and solutions used in this study

The composition of solutions and buffers used in this study are either described in the relevant sections or in Appendix 2.

2.3.3 Determination of bacterial eggshell contamination

After egg samples were selected, the next step was to isolate bacteria from the eggshell and plate them on Plate Count Agar (PCA). The treatment method used was similar to a method performed by Gentry and Quarles (1972). The method involved picking up an egg with sterile tweezers, and placing it in a sterile bag containing 10 ml of 1x Phosphate Buffered Saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.0). The bag was held at an angle with the egg and diluent in the corner. Then, the egg was rubbed thoroughly for 20 seconds in order to detach bacteria from the eggshell surface. The suspensions were successively diluted up to 10^{-5}, by inoculating 1 ml of the egg rinse in 9 ml 1x PBS (Figure 2.1). Each dilution performed was followed by plating 100 µL of the suspension on Plate Count Agar (PCA, Oxoid) for bacterial enumeration, and was plated in triplicate. The plates were incubated for 72 h at 30 °C. Isolation of selective pathogens was also performed on different selective media at a dilution of 10^{-1} as described in Table 2.1(Further details in section 2.3.6). The total viable counts (TVCs) of bacteria isolated from the eggshell were calculated by counting the colony forming units (CFU)/eggshell. The formula used for calculating CFU/eggshell was as the following equation:

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL) x total dilution used}} \rightarrow \frac{\text{Number of CFU}}{\text{mL}}$$

The TVCs CFU/ml was multiplied by 10 to convert the TVCs to CFU/eggshell. A control sample was performed in each treatment session to ensure that the materials used were sterile.

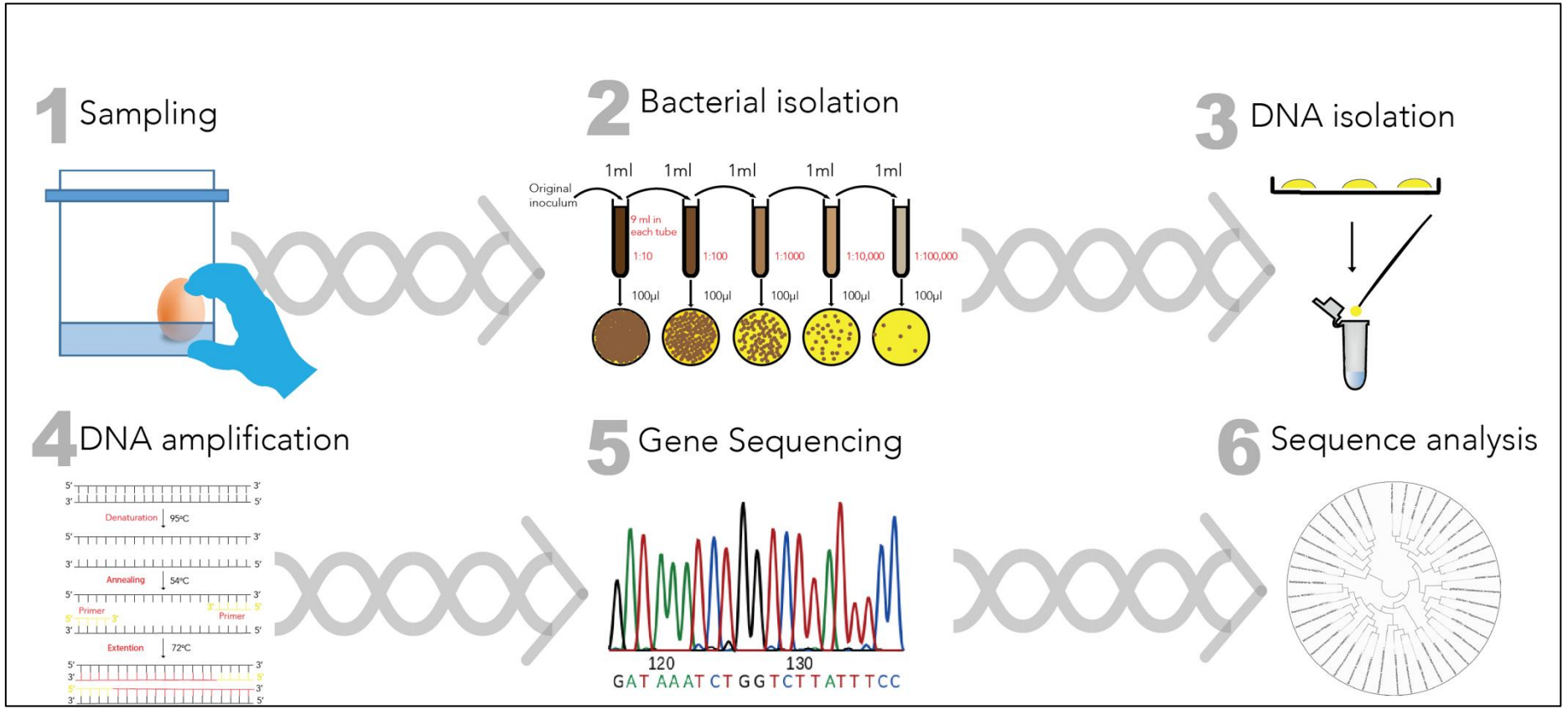

Figure 2.1 The main work flow used, for isolating bacteria from table eggs, and identifying the isolates by sequencing part of the 16S rRNA gene. The work flow describes 6 main stages of the analysis, starting from the sampling step and ending with analysis of the sequences. Each image represents the method used for the analysis.

Table 2.1 Culture media and technical procedures used for isolating bacteria from table eggs. For some selective media, a supplement was added according to the manufacturer's instruction.

Microorganisms	Culture medium	Culture technique	Incubation		Medium Product	Company
			Temp (°C)	Time (h)		
Aerobic bacteria	PCA[a]	Spread plate 100 µL	30	72	CM0325	Oxoid
Staphylococcus	BPA[b] +[c]Selective supplement	Spread plate 100 µL	37	48	CM0961 SR0122A	Oxoid
Salmonella	BGA[d]	Spread plate 100 µL	37	48	CM0329	Oxoid
E.coli	EMBA[e]	Spread plate 100 µL	37	48	CM0069	Oxoid
Campylobacter	CCDA[f]+[g]Selective supplement	Spread plate 100 µL (anaerobic incubation)	40	48	CM0739 SR0155E	Oxoid
Listeria	LSA[h]+[i]Selective supplement	Spread plate 100 µL	37	48	CM0856 SR0206E	Oxoid
Clostridium	RCA[j]	Spread plate 100 µL (anaerobic incubation)	37	48	CM0151	Oxoid

[a]Plate count agar; [b]Baird Parker agar; [c]Pancreatic digest of casein, Meat extract, Yeast extract, Sodium pyruvate, Lithium chloride; [d]Brilliant Green Agar; [e]Eosin Methylene Blue agar; [f]*Campylobacter* blood-free selective agar; [g]Amphotericin and Cefoperazone; [h]*Listeria* selective agar; [i]Colistin sulphate, Cycloheximide, Acriflavine and Cefotetan; [j]Reinforced Clostridial agar.

2.3.4 Determination of bacterial egg content contamination

For isolating bacteria from egg content, a different treatment technique was developed. Before evacuating egg content, the eggshell was decontaminated by soaking in 70% ethanol for 5 seconds, followed by flaming for 2 seconds, and then drying in a laminar flow cabinet for 5 minutes as described by Himathongkham *et al*. (1999). This pre-treatment was performed to avoid any cross contamination that might occur from the eggshell to egg content. The test was performed on the same eggs that were previously treated for determining the level of contamination on eggshell. A control sample to ensure sterility of the eggshell was performed by plating eggshell rinse on PCA, and incubating for 72 h at 30 °C. Then, the egg was aseptically cracked in a sterile plastic bag using a sterile blade.

The egg content sample was homogenised in a Stomacher®400 (Seward) for 30 seconds to mix egg albumin with the egg yolk. After the sample was homogenised, 25 g of the homogenate was transferred to another sterile plastic bag, and mixed with 225 ml of 1x PBS. Afterwards, the sample was homogenised in the Stomacher®400 for 2 minutes in order to release bacteria from the aggregated protein. The suspension was then successively diluted up to 10^{-5}, and 100 µL of dilutions were plated on PCA. The plates

were incubated at 30 °C for 72 h. After bacteria had grown on the plate, the TVCs were calculated using the following formula equation.

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL) x total dilution used}} \rightarrow \frac{\text{Number of CFU}}{\text{mL}}$$

The modification in this method lies in homogenizing egg content with diluent buffer using the Stomacher to detach bacteria from egg proteins, instead of mixing egg homogenate by hand as described in previous studies (Himathongkham *et al*., 1999; De Reu *et al.*, 2006b)

2.3.5 Morphological characteristics of bacterial isolates

After bacteria had grown on different agar media, the next step was to study their colonies and cell morphology. For this, bacterial colonies were selected at random, based on their morphological variations. Several characteristics were considered when selecting isolates, including colony shape, colour and size. The isolates were sub-cultured on fresh PCA agar to perform molecular analysis using, and their morphological characteristics were studied. To study the cell morphology of a single isolate, a Gram stain method was conducted. A heat-fixed smear was performed by using a sterile loop, to smear the sample on a microscope slide, which was stained by 0.3% crystal violet dye for 1 min, and rinsed with ddH_2O. 1% iodine was then flooded on to bind the dye for 30 sec, and rinsed with ddH_2O. A solution of 1:1 ethanol: acetone was used to remove the excess stain for 30 sec, and the sample was then rinsed with water. Finally, the slide was stained with safranin for 1 min and rinsed with ddH_2O. The cell morphology was examined under the microscope.

2.3.6 Isolation of table egg bacteria on selective media

For the purpose of providing the growth requirements, oxygen conditions and appropriate temperature for particular microbes to grow, the eggshell rinse and egg content homogenate of a representative samples of 16 eggs were plated on different selective media as described in Table 2.1 In order to isolate and obtain the TVCs of *Staphylococcus* bacteria, 100 µL of the 10^{-1} dilution from the eggshell wash and egg content homogenate

were plated on Baird-Parker agar and incubated at 37 °C for 48 h. Similarly, for isolating *E. coli*, 100 µL of the 10^{-1} dilution were plated on Eosin-Methylene Blue agar, and incubated at 37 °C for 48 h. Also, for isolating *Campylobacter* spp. the suspension was plated on *Campylobacter* blood-free selective agar and placed in an anaerobic jar containing a gas generating system (CampyGen, Oxoid, UK). The system produces a gas mixture of 5 % oxygen, 85 % nitrogen, 10% carbon dioxide. For isolating *Salmonella*, 100 µL of the suspension of the first dilution 10^{-1} were plated on Brilliant Green Agar (BGA), and for *Listeria* samples were plated on Listeria Selective Agar (LSA). The plates were incubated at 40 °C for 48 hrs. For isolating *Clostridium* bacteria 100 µL of the suspension was plated on Reinforced Clostridial Agar (RCA), and incubated in anaerobic cabinet containing a gas mixture of 10 % hydrogen, 80 % nitrogen, 10 % carbon dioxide, the plates were incubated at 37 °C for 48 hrs. Enrichment steps were not used in the selective isolations as this would have influenced the relative numbers of organisms detected by the analysis.

2.3.7 Genomic DNA extraction

The selected pure cultures were subjected to DNA extraction to identify the bacteria. Cell lysate including DNA was extracted using the rapid boiling method, which involved picking a single colony from sub-culture pure isolates as described in section 2.3.5. The colony was mixed with 20 µl sterilised ddH_2O by vortexing. Then, the suspension was incubated at 100 °C for 10 minutes, and was placed on ice for 3 minutes, and finally centrifuged for 3 minutes at 13,000xg to pellet the cell debris. The lysate was incubated on ice, and used the same day. All DNA templates were prepared from pure cultures that were restreaked to provide single colonies.

2.3.8 Primer selection and PCR amplification conditions

Identification of bacterial isolates was performed through using a PCR that targeted part of 16S rRNA gene. The 16S rDNA primers used in this study were 9F (5′-GAGTTTGATCCTGGCTCAG-3′; position 9-27, *Escherichia coli* 16S rRNA numbering) and 536R (5′-GTATTACCGCGGCTGCTG-3′; position 536-519). This primer was previously utilised by Kim *et al*. (2004) for detection of bacterial isolate from water samples. Also, it has been suggested that for most bacterial isolates the initial 500-

bp sequence provides adequate differentiation for identification (Clarridge, 2004). PCR was performed in a 50 µl reaction mix containing 25 µl 2x BioMix buffer (Bioline), 1µl (100 µM 27F forward primer), and 1µl reverse primer (100 µM 536R reverse primer), and 2 µl DNA template (50-60 ng). The PCR reaction mixture was heated at 95 °C in a thermal cycler for 4 min, and the PCR program was set on 30 reaction cycles of 95 °C for 30 sec; followed by annealing step at 54 °C for 30 sec; then elongating step at 72 °C for 30 sec; and final extension at 72 °C for 7 min. The cycle was limited to 30 reactions. The melting temperatures of the primers were calculated using the formula T_m = (G+C) 4 + (A+T) 2, and the PCR annealing temperature was calculated using the formula (T_m-5).

2.3.9 PCR band visualisation on (1%) agarose gel

The PCR products were run on a 1% (w/v) agarose gel to detect whether the amplifications were successfully performed, as described by Corthell (2014). Agarose gel powder (Bioline) was dissolved to a concentration of 1% (w/v) in 1xTris-acetate EDTA buffer (TAE). The mixture then was heated in a microwave for 1 minute to completely melt the agarose powder in the buffer. After cooling 1 µl of 10 mg/ml ethidium bromide was added, and the gel was poured into a gel container sealed with autoclave tape at the edges. A comb was inserted into the gel to form sample wells. After the gel had solidified, the comb and the tape were removed, and the gel was immersed in 1 x TAE buffer. To visualise PCR products on the gel, 5 µl of the products was mixed with 2 µl of 5x loading buffer (Bioline), and loaded on to the gel. 5 µl of hyper ladder I (Bioline) was loaded alongside the samples to estimate the size of amplified DNA (Figure 2.2). The gel was run in 1x TAE buffer at 50 V until the loading dye reached the marked line on the gel tray. DNA bands were detected using a doc-UV cabinet (Bio-Rad). Image lab 4.1 software (Bio-Rad) was used to edit and produce the image, and an illustration of the gel was performed using the illustrator CS6 software (Adobe).

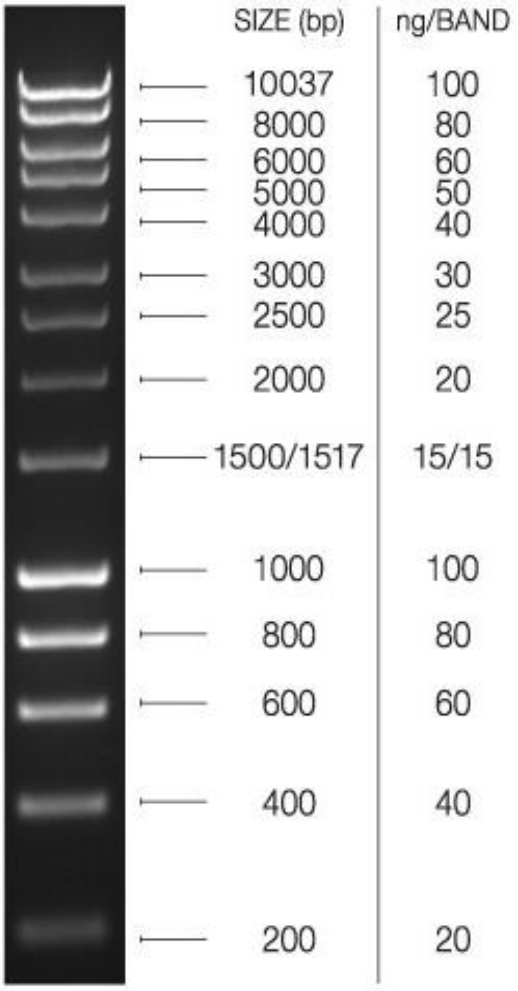

Figure 2.2 Hyper Ladder I (Bioline).

2.3.10 Partial 16S rRNA gene sequencing

The next procedure, after bacterial DNA had been amplified and visualised on a gel, was DNA purification to remove dNTPs, excess of primers and other impurities. Therefore, PCR products were purified using a QIAquick spin column (Qiagen), and the procedures were followed according to the manufacturer's instructions. The procedures performed were based on 3 main steps starting by binding DNA to silica membrane in the spin column, then DNA was washed with ethanol and finally bound DNA was eluted using 50 µl of TE buffer (pH-8.0). In preparation for sequencing 1-5 µg of purified DNA and 1 µl (5 pmol/µl) of each primer were made up to a final total volume of 6 µl using Nuclease-Free Water (Thermo). All DNA samples in this study were sequenced by the GenePool group in University of Edinburgh (http://genepool.bio.ed.ac.uk).

2.3.11 Analysis of 16S rRNA gene sequence

Sequencing chromatograms were visualised using 4 Peaks version 1.8. Then, the forward sequence was aligned with reverse sequence using the align tool in the NCBI website; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq. After the sequences were aligned, they were blasted using the tool BLASTN from the NCBI website, to determine phylogenetic similarities with bacterial strains in the GenBank sequence database.

2.3.12 Data analysis

A Pearson-coefficient correlation was applied to show the relationship between TVCs of eggshell and TVCs of egg content. One-way ANOVA test was performed to reveal if there was any significant difference between TVCs and different housing systems. All statistics were conducted using IBM SPSS®20.

2.4 Results

In this study culture and molecular approaches were used to identify bacterial isolates from eggs. Isolating bacteria from eggshell rinse and egg content homogenate were performed on plate count agar (PCA), to obtain the TVCs, while samples also plated on different selective media for targeting particular bacteria. Also, DNA isolation and sequencing of part of the 16S rRNA were conducted to identify additional bacteria present which may contribute to egg spoilage.

2.4.1 Total viable counts of bacteria isolated from eggshell and egg content

For the purpose of determining bacterial contamination of table eggs, it was of interest to estimate the total viable counts (TVCs) of bacteria. During the study, 88 table eggs collected from different sources as described in Appendix I were prepared for microbial analysis. All eggs were grade A and were obtained through purchase from local market in Edinburgh and their dates were still valid for human consumption, and the eggs represented different housing systems to allow for comparisons to be made. The result obtained showed that the TVCs from eggshell were variable. The washing of intact eggs in diluent buffer by rubbing them in a sterile bag was found to be the most effective method in terms of recovering bacteria (De Reu *et al*. 2009). A total of 176 samples including eggshell and content were tested for TVCs. Each sample suspension was cultured on plate count agar and incubated at 30 °C for 72 h.

The result revealed free-range eggshell had the highest microbial load with an average TVCs of 5.7×10^5 CFU/eggshell, followed by organic free range with an average TVCs of 2.9×10^5 CFU/eggshell and finally caged eggs accounted for 2.0×10^5 CFU/eggshell (Figure 2.3). Moreover, the result showed multiple outliers in the TVCs of organisms isolated from the eggshell; thus, it is clear that the TVCs isolated from eggshell are variable between the collected samples. For testing the significance of the difference between the mean of TVCs of the 3 different sources, ANOVA-test was performed. As can be seen from Table 2.2 The result showed that there is no significant difference ($p = 0.141$), between the egg sources, in terms of the TVCs. Also, p-value was found to be not significant ($p = 0.06$) in comparing TVCs of free range egg and caged eggs.

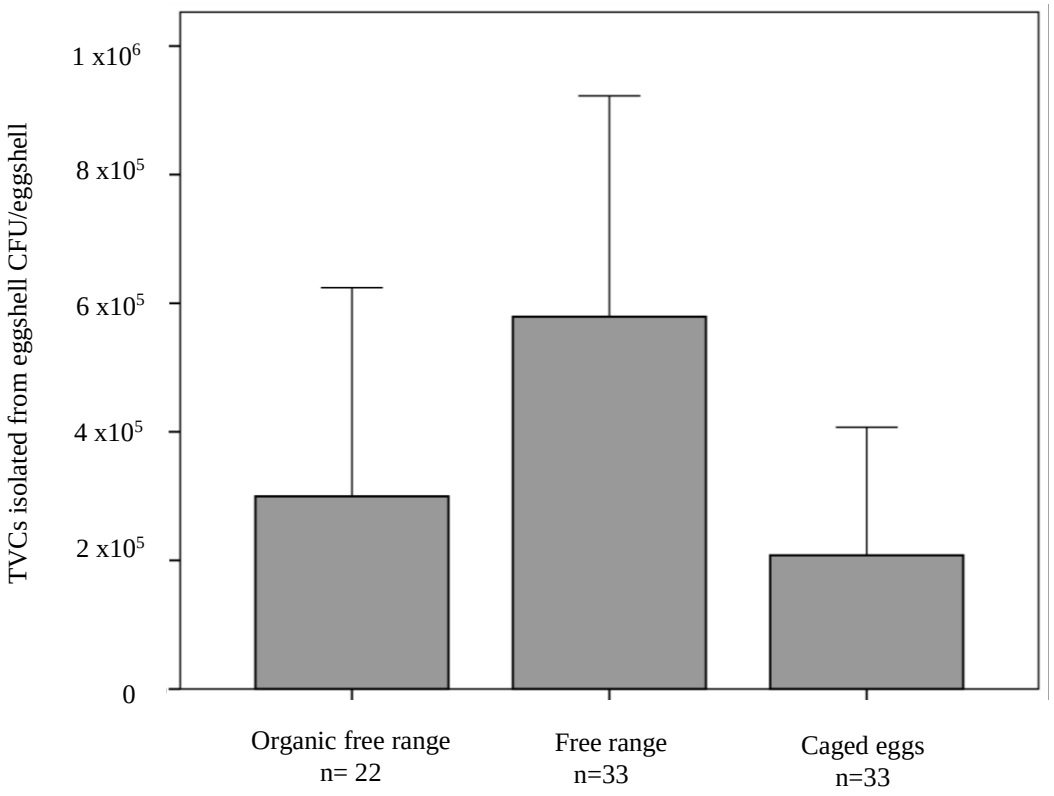

Figure 2.3 Total viable counts of bacteria isolated from different types of eggshell. T-bar indicates standard errors.

De Reu *et al*. (2009) found that the average TVCs of eggshell within six furnished cages were ranging from 1.7 x 10^4 to 3.2 x 10^5 CFU/eggshell, whereas the TVCs obtained from eggshell of six non-cage systems were ranging from 2.2 x 10^4 to 1.6 x 10^5 CFU/eggshell. The result obtained in this study in terms of level of contamination from caged and non-caged systems were in agreement with the result recorded by De Reu *et al*. (2009).

Table 2.2 Total mean of bacterial counts from egg shell of different housing systems.

H.S[a]	N[b]	Mean CFU[c]/eggshell	S.D[d]	F[e]	Sig.[f]
ORFE[g]	22	299436	732306	2.0	.141
FR[h]	33	579142	968274		
CE[i]	33	207738	562360		
Total	88	369939	786417		

[a]Housing system; [b]Number of eggs; [c]Colony Forming Units; [d]Standard deviation; [e]F-value; [f]Significant difference; [g]Organic Free range; [h]Free range; [i]Caged egg.

Regarding egg content, the results revealed that organic free-range eggs had the highest TVCs (1.7 x 10^3 CFU/ml), followed by the free-range eggs (1 x10^3 CFU/ml), with the lowest count obtained from the caged system (4.2 x 10^2 CFU/ml) (Figure 2.4). As can be seen from table 2-3 that ANOVA- test showed no significant different between the TVCs isolated from egg content obtained from different sources (p=0.59).

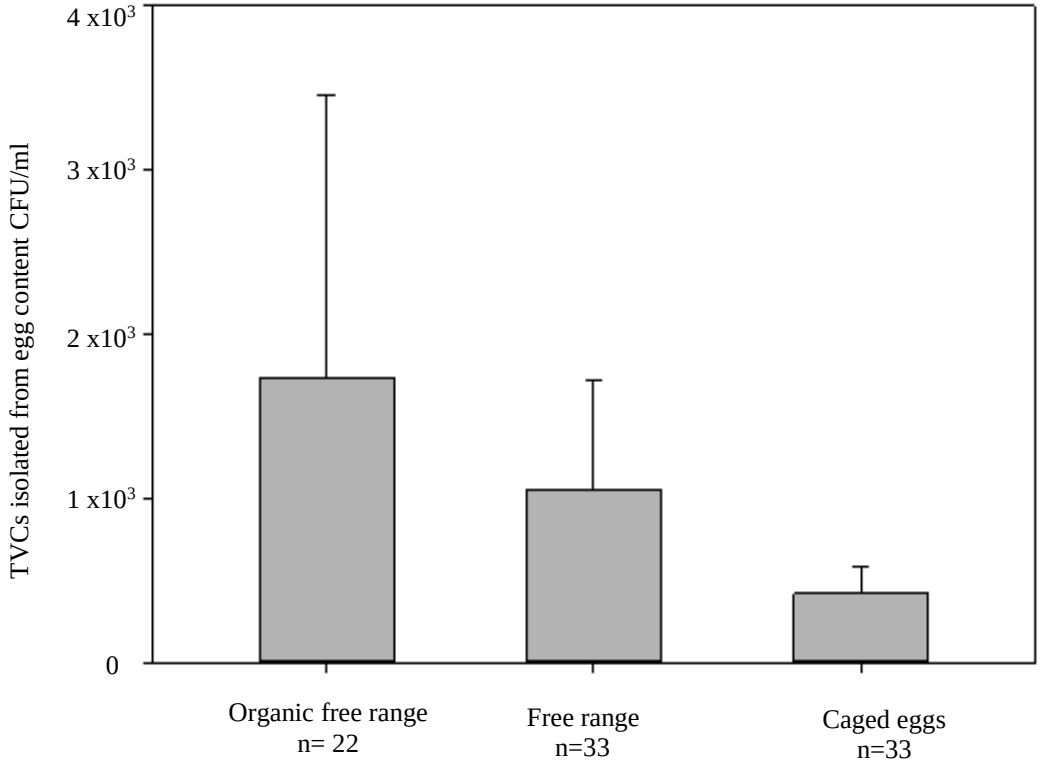

Figure 2.4 TVCs isolated from egg content of different sources of table eggs. T-bar indicates standard errors.

Table 2-3 Total mean of bacterial counts from egg content of different housing systems.

H.S[a]	N[b]	Mean CFU[c]/eggshell	S.D[d]	F[e]	Sig.[f]
ORFE[g]	22	1736	8099	0.51	0.59
FR[h]	33	1052	3867		
CE[i]	33	422	920		
Total	88	986	4681		

[a]Housing system; [b]Number of eggs; [c]Colony Forming Units; [d]Standard deviation; [e]F-value; [f]Significant difference; [g]Organic Free range; [h]Free range; [i]Caged egg.

Free range eggs are expected to contain higher levels of contamination than caged eggs, since hens in caged systems lay their eggs on clean surfaces, compared to free range systems where hens have access to outdoor and they may lay their eggs in the soil. The level of variability in the TVCs of both eggshell and egg content suggests that bacterial counts differ from one sample to another. However, this variability could be due to many factors, including that hens are infected with bacteria, eggs being laid on contaminated surfaces or cross-contaminations occuring during the handling process (De Reu *et al*., 2008; Gast and Holt, 2000). Moreover, bacterial contamination of the egg content could result from the penetration of the shell by bacteria deposited on the surface of the egg (Harry, 1963).

2.4.2 Morphological characterisation of PCA isolates and identifying the isolates based on 16S rRNA gene analysis

After bacteria were isolated on PCA, different colony morphologies were observed, and when selecting bacterial isolates, several factors were considered to discriminate between isolates, including colour, shape and size of bacterial colonies. The next step was to select these isolates as described in section 2.3.5, and sub-culture them on fresh PCA agar media, so as to perform further identification tests using the 16S rRNA gene.

The majority of the isolates on PCA had similar morphological characteristics; circular in shape, entire margin, smooth surface and white to creamy colour (Figure 2.5, A). However, a few isolates had distinct colours as shown in Figure 2.5, B, or irregular shape and undulate margin as can be seen in Figure 2.5, C, while some had a glistening surface as shown in Figure 2.5, D.

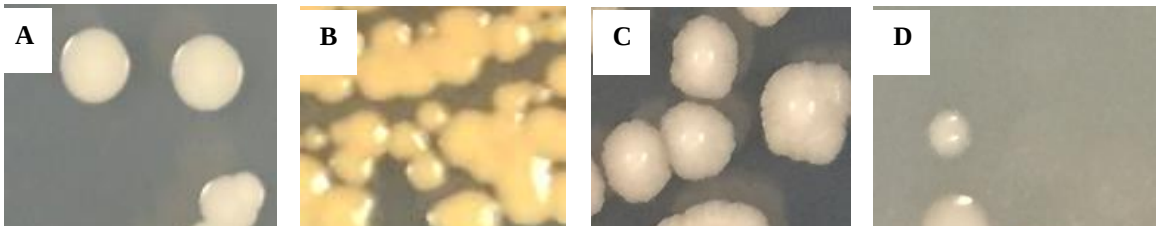

Figure 2.5 Colony morphologies of bacteria isolated from the eggs, A: *Staphylococcus equorum* (C3) ; B: *Micrococcus luteus* (C1), C: *Bacillus safensis* (C20), D: *Acinetobacter lwoffii* (C45). All the isolates were plated on plate count agar, and incubated at 30 °C for 72 h.

A total of 47 bacterial isolates were selected from PCA, in order to identify the bacteria. As mentioned in the introduction, limitations of using the 16S rRNA gene as an identification tool are in the time that is required to prepare a sample for sequencing and analysing the result of sequences, as well as the high cost of using this method compared to others. Thus, the bacterial isolates were selected at random based on the variations explained earlier to obtain a set of isolates that may describe the bacterial community of table eggs. The 47 isolates selected represented different isolates that were observed on PCA based on their morphological characteristics. Selected strains were subjected to a DNA extraction, then the DNA fragments were amplified using PCR and screened on an agarose gel to confirm presence of the PCR products. The isolates were identified by sequencing part of the 16S rRNA gene. The length of the amplified rDNA sequences was in agreement with the expected size, around 590 bases (Figure 2.6).

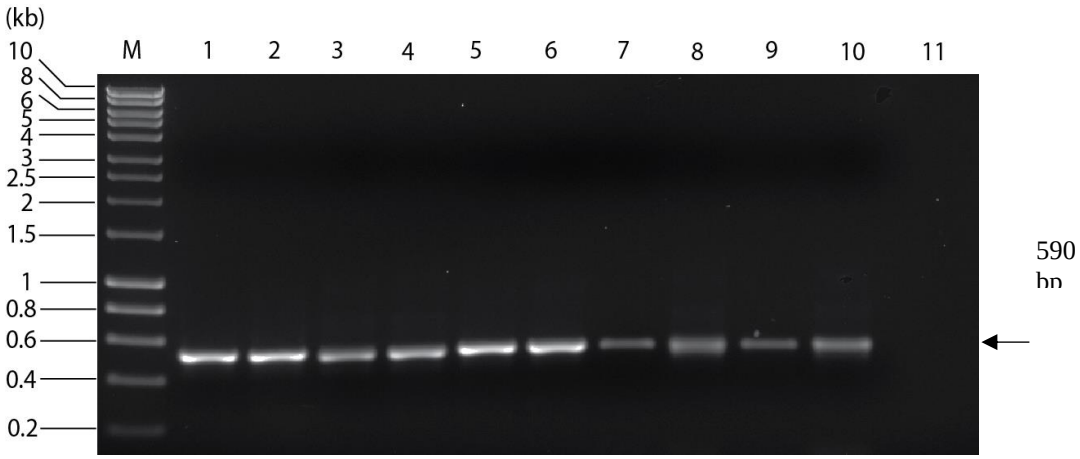

Figure 2.6 Agarose gel electrophoresis (1%) showing amplified DNA amplicons derived from single bacterial colonies. Lane M: hyper ladder I, lane 1-10 from bacterial DNA amplicons, and lane 11 negative control sample.

All sequences were subjected to a bioinformatics analysis, by using Genbank BLAST search, which identifies the sequence by identity to those in the database. The results are illustrated in Table 2.2. A total of 34 sequences showed identity of equal or more than 99 % compared to gene sequences in the NCBI database. The isolate C18 had an identity of 91%, to an uncultured bacterial clone. This isolate had morphological characteristics that are similar to *Micrococcus luteus* (C11).

Table 2.4 The bacterial sequences isolated from table eggs, describing the morphological characteristics of each isolate, source of isolation, similarity percentage, the accession number in GenBank and egg source.

Isolate	Culture Characteristics						Sequencing result	Source	N.[a] match in %	GenBank Accession No.	Egg Source
	Shape	Margin	Elevation	Surface	Colour	Gram stain					
C1	Circular	Entire	Raised	Smooth	Yellow	+ve	*Micrococcus luteus*	Shell	99%	LK020770	CE[b]
C2	Circular	Entire	Raised	Smooth	White	+ve	*Staphylococcus saprophyticus*	Shell	99%	KF792262	CE
C3	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	100%	EU665637	CE
C4	Circular	Entire	Raised	Smooth	Pale yellow	-ve	*Stenotrophomonas maltophilia*	Shell	99%	GU726589	ORGF[c]
C5	Circular	Entire	Convex	Smooth	Yellow	-ve	*Brevundimonas bullata*	Shell	97%	JQ595507	ORGF
C6	Circular	Entire	Convex	Smooth	Yellow	-ve	*Brevundimonas bullata*	Shell	99%	KP072753	ORGF
C7	Circular	Entire	Convex	Smooth	Yellow	-ve	*Brevundimonas bullata*	Shell	99%	EU734663	FRE[d]
C8	Circular	Entire	Raised	Smooth	Yellow	-ve	*Stenotrophomonas rhizophila*	Shell	99%	HQ327141	CE
C9	Circular	Entire	Raised	Smooth	Cream	-ve	*Stenotrophomonas maltophilia*	Shell	99%	KF254518	CE
C10	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	99%	HG941668	FRE
C11	Circular	Entire	Raised	Smooth	Yellow	+ve	*Micrococcus luteus*	Shell	99%	KF600756	FRE
C12	Irregular	Undulate	Flat	Rough	White	+ve	*Bacillus flexus*	Content	99%	KR809411	CE
C13	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	99%	KP224447	CE
C14	Circular	Entire	Flat	Smooth	Yellow	+ve	*Staphylococcus lentus*	Shell	100%	FJ002279	CE
C15	Circular	Entire	Raised	Smooth	Yellow	+ve	*Micrococcus luteus*	Shell	100%	KT339390	FRE

C16	Circular	Entire	Raised	Smooth	Yellow	+ve	*Staphylococcus epidermidis*	Shell	99%	JX067904	FRE
C17	Circular	Undulate	Raised	Smooth	White	+ve	*Brachybacterium paraconglomeratum*	Shell	99%	JQ712514.1	FRE
C18	Circular	Entire	Raised	Smooth	Yellow	+ve	Uncultured bacterium clone	Shell	91%	KC581675	CE
C19	Circular	Entire	Raised	Smooth	Yellow	+ve	*Kocuria palustris*	Content	97%	HE716941	CE
C20	Irregular	Undulate	Raised	Rough	White	+ve	*Bacillus safensis*	Content	99%	JF836885	ORGF
C21	Circular	Entire	Raised	Smooth	Yellow	+ve	*Micrococcus luteus*	Content	97%	KF600756.1	ORGF
C22	Circular	Entire	Raised	Smooth	Yellow	+ve	*Kocuria atrinae*	Shell	97%	NR_116744	ORGF
C23	Circular	Entire	Raised	Smooth	Opaque	-ve	*Acinetobacter lwoffii*	Shell	96%	HE651921	CE
C24	Circular	Entire	Raised	Smooth	Yellow	-ve	*Brevibacterium epidermidis*	Shell	97%	KJ575062	CE
C25	Circular	Entire	Raised	Smooth	White	+ve	*Staphylococcus lentus*	Shell	98%	FJ795656	FRE
C26	Circular	Entire	Flat	Rough	White	+ve	*Staphylococcus saprophyticus*	Shell	98%	KF906833	FRE
C27	Circular	Entire	Raised	Smooth	Yellow	+ve	*Staphylococcus caprae*	Content	98%	HG421011	CE
C28	Circular	Entire	Raised	Smooth	Cream	+ve	*Micrococcus luteus*	Content	100%	HF562858	CE
C29	Circular	Entire	Raised	Smooth	Yellow	+ve	*Micrococcus luteus*	Content	99%	HE575913	ORGF
C30	Circular	Entire	Raised	Smooth	Cream	-ve	*Moraxella osloensis*	Content	99%	KC456542	FRE
C31	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Content	100%	HG941668	ORGF
C32	Circular	Entire	Raised	Smooth	Yellow	-ve	*Chryseobacterium hominis*	Content	98%	AM423087	FRE
C33	Circular	Entire	Raised	Smooth	Cream	+ve	*Micrococcus luteus*	Shell	99%	KF054946	FRE

C34	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	95%	JX315320	FRE
C35	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	99%	KM036089	ORGF
C36	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	99%	HE651910	ORGF
C37	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	99%	JX315320	ORGF
C38	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	99%	EU855190	FRE
C39	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	99%	JX077101	CE
C40	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	99%	EU855190	FRE
C41	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	99%	KP224447	FRE
C42	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	99%	KJ862003	FRE
C43	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus equorum*	Shell	99%	JX315320	CE
C44	Circular	Entire	Raised	Smooth	White	+ve	*Staphylococcus equorum*	Shell	99%	KR012324	CE
C45	Circular	Entire	Flat	Smooth	Opaque	-ve	*Acinetobacter lwoffii*	Shell	96%	HE651921	CE
C46	Circular	Entire	Raised	Smooth	Cream	+ve	*Nocardiopsis alba*	Shell	99%	KC989931	CE
C47	Circular	Entire	Raised	Smooth	Cream	+ve	*Corynebacterium ammoniagenes*	Shell	99%	HE858280	CE

[a] Nucleotid match; [b]caged egg; [c]organic free range egg; [d]free range egg. The medium used for the isolation was plate count agar.

The most frequent isolates that appeared on PCA had colonial morphologies of white to creamy colour with circular shape, entire margin and smooth surface. From the 47 isolates selected for sequencing 24 isolates had this colony morphology. Gram staining was performed on isolated colonies and cells were observed under the microscope. Generally, 36 isolates were Gram +ve and 11 were Gram negative, with 32 Gram positive cocci, 3 Gram positive rods and one Gram +ve filamentous organism. In terms of Gram negative, 9 were rods and 2 were coccobacilli. All Gram-staining behaviour of the isolates agreed with the identification results by using the 16s rRNA gene. 21 isolates were identified from the eggshell and identified to belong to the genera of *Staphylococcus* (17), *Corynebacterium* (1), *Nocardiopsis* (1), *Micrococcus* (1) and *Stenotrophomonas* (1). From egg content 3 isolates were identified to belong to the genera of *Staphylococcus* (1), *Micrococcus* (1), *Moraxella* (1).

Among the 17 *Staphylococcus* isolates identified from the eggshell, there was a high occurrence of *Staphylococcus equorum* compared to other *Staphylococcus* species. In another study by De Reu *et al*. (2006), *S. equorum* was also isolated from eggshell and identified in high abundance. It is found in mammalian skin as part of normal flora, and considered to be of low virulence with resistance to antibiotics (Nováková *et al.*, 2006). Moreover, *S. equorum* was isolated from soil, water, skin, cheese, sausage, and frequently from fermented food (Leroy *et al.*, 2009). It was isolated in this study from both eggshell and egg content.

The second most common isolates that appeared on PCA had colony morphologies of yellow colour, circular shape, entire margin and smooth surface. This colony morphology formed 18 of the total 47 isolates selected for sequencing, 13 were isolated from the egg shell and identified to belong to the genera *Micrococcus* (3), *Brevundimonas* (3), *Stenotrophomonas* (2), *Staphylococcus* (2), *Brevibacterium* (1), *Kocuria* (1) and Uncultured bacterium isolate (1). From egg content 5 isolates were identified to belong to the genera of *Micrococcus* (3), *Staphylococcus* (1) and *Kocuria* (1). Of the total 18 isolates that were identified as *Micrococcus luteus* using the 16S rRNA sequencing, 7 isolates were had morphological characteristics of yellow colour, circular shape, entire margin and smooth surface. A study by Chaemsanit *et al*., (2015) found *Micrococcus luteus* was the second major contaminant from eggshell. *M. luteus* has been isolated from

soil, skin, sewage water and marine biofilms (Vimalanathan *et al*., 2013; Clark *et al*., 2000)

The next distinct colony morphology that appeared on PCA was of two colonies that had irregular shape, undulate margin and the colour varied from white to creamy. These isolates from egg content had an identity to the genus *Bacillus*. The remaining isolates were showing single colonial morphologies varied between the morphological characteristics described earlier. All bacteria examined belonged to one of 13 bacterial genera (*Staphylococcus, Micrococcus, Brevundimonas, Stenotrophomonas, Bacillus, Brevibacterium, Corynebacterium, Actinobacter, Brachybacterium, Chryseobacterium, Kocuria, Nocariopsis, and Moraxella*).

Most of the isolates are normal flora that can be found in the environment, on skin and in foods. Nevertheless, a small number of these isolates have been associated with clinical pathogens. *Staphylococcus epidermidis* was found to cause endocarditis, most often in patients with a defective heart valve (Karchmer *et al*., 1983). It has also been reported to cause sepsis in a patient using a catheter for parenteral nutrition (Sitges-Serra *et al*., 1980). *Acinetobacter lwoffii* is another pathogen that is present on skin as normal flora, but it has been associated with catheter infection in immunocompromised patients (Ku *et al*., 2000). It was also responsible for human gastroenteritis (Regalado, Martin and Antony, 2009). More importantly, *Acinetobacter* spp. are resistant to most available antibiotic agents (Manchanda, Sanchaita and Singh, 2010).

The genera *Micrococcus, Staphylococcus, Acinetobacter*, *Aeromonas*, *Corynebacterium*, *Klebsiella* and *Stenotrophomonas* have been isolated from table eggs in previous studies (Ayres *et al.*, 1966; Schwaiger, Schmied and Bauer, 2010; Ruiz-de-Castañeda *et al.*, 2011; Potter *et al.*, 2013). However, other bacterial genera, including *Chryseobacterium, Kocuria, Brevundimonas, Nocardiopsis, Brevibacterium* and *Brachybacterium* have been isolated from other sources, but none of them have been isolated previously from table eggs.

Of the 88 egg samples tested, enteric bacteria that are responsible for food poisoning were not identified from either of the fractions of eggs by using the extraction methods described in sections 2.3.3, and 2.3.4, and culturing on PCA.

2.4.3 Isolation of bacteria from table eggs on different selective media

After determining the general characteristics of bacterial contamination by culturing on PCA, the next step was to attempt to isolate specific bacteria using selective media. A number of 16 samples were tested in which egg rinse from eggshells and the egg content homogenate were spread on varied selective media including; Baird-Parker agar (BPA) for targeting *Staphylococcus* sp., *Listeria* selective agar (LSA) for targeting *Listeria*, Reinforced Clostridial agar (RCA) for targeting clostridia, Brilliant Green Agar (BGA) for targeting *Salmonella* sp., Eosin Methylene Blue agar (EMBA) for targeting Gram negative bacteria and CCDA for targeting *Campylobacter* sp. Plates were incubated as described in the materials and methods section.

Determining the TVCs of *Staphylococcus* on selective media may provide an estimate of *Staphylococcus* compared to the TVCs that were determined on PCA. The results showed that microbial counts from eggshell samples cultured on BPA had the highest bacterial counts with mean of 3.9×10^3 CFU/eggshell compared to TVCs obtained on other selective media (Table 2.3). As mentioned previously, *Staphylococcus* has been found to be the major contaminant of the eggshell in different studies (De Reu *et al*. 2008; Chaemsanit *et al*. 2015). The microbial counts of *Staphylococcus* sp from the eggshell were estimated to be 6×10^2 CFU/cm^2 (Alvarez-Fernández *et al.*, 2012). In another study, 66.67 % from a total of 150 eggshells were positive for *Staphylococcus* sp. (Fardows *et al.*, 2016).

Table 2-5 Viable counts from both eggshell and egg content using different selective media

Agar	Targeted Organism	TVCs eggshell CFU/eggshell [a](*n*=16)	[b]S.D.	[c]S.E.	TVCs egg content CFU/ml (*n*=16)
BPA[d]	*Staphylococcus*	3.9×10^3	4723	1181	0
LSA[e]	*Listeria*	3.1×10^1 *	125	31	0
RCA[f]	*Clostridium*	9×10^2 *	2156	539	0
BGA[g]	*Salmonella*	0	0	0	0
EMBA[h]	Coliform	0	0	0	0
CCDA[i]	*Campylobacter*	0	0	0	0

Values are CFU per eggshell, and CFU/ml for egg content, [a] number of eggs treated; [b]Standard deviation, [c]Standard error, [d]Baird-Parker agar; [e]*Listeria* selective agar; [f]Reinforced Clostridial agar; [g]Brilliant Green

agar; [h]Eosin Methylene Blue agar; [i]*Campylobacter* blood-free selective agar. ∗ An average TVCs of bacterial growth detected on LSA and RCA agars, for *Listeria* only one plate showed growth and the isolates were found to be *Staphylococcus haemolyticus* and for *Clostridium* 3 plates showed growth of *Clostridium perfringens.*

A total of 6 isolates were selected for 16S rRNA analysis, and the results showed that all isolates belonged to the genus *Staphylococcus* (Table 2.4). This indicates that BPA has suppressed growth of other bacteria, and therefore the TVCs that were estimated on BPA represent the counts of *Staphylococcus* bacteria. Of the 6 isolates, 5 (SE1, SE2, SE3, SE5 and SE6) had identity of ≥ 98 % to *Staphylococcus equorum*, whereas the other isolate SE4 had identity of 100 % to *Staphylococcus caprae*. Both *S. equorum* and *S. caprae* were previously isolated on PCA plates. The *S. equorum* occurred more frequently in both analyses, confirming the high occurrence of *S. equorum* on eggshell. *Staphylococcus* was isolated in a previous work in this study from the egg content using PCA, but it is surprising that was not isolated on a selective medium BPA.

In terms of *Listeria*, the samples average counts on LSA plates were 3.1×10^1 CFU/eggshell, however, out of 16 sample tested only one plate showed growth with the count of 5×10^2 CFU/eggshell. The morphological characteristics of the colonies isolated were identical to each other. The colonies appeared to be creamy in colour, circular in shape with an entire margin as shown in Figure 2.7. However, according to the medium manual, *Listeria* should appear on the plate as a gray colony that hydrolyses aesculin, producing a black zone around the colony due to formation of black iron phenolic compounds. After performing DNA analysis on one of the isolates, the result showed that isolate SE10 had identity of 99% to *Staphylococcus haemolyticus* (Table 2.4). It is stated in the medium manual that most Gram-positive species are suppressed, but some strains of *Staphylococcus* may grow as aesculin-negative colonies. Thus, from the total number of samples tested, it appeared that no *Listeria* spp were identified from eggshell or egg content.

Table 2-6 Bacteria isolated from table eggs on different selective media, describing the morphological characteristics of each isolate, source of isolation, similarity percentage, the accession number in GenBank and the medium used for the isolation.

Isolate	Culture Characteristics						Sequencing result	Source	N.[a] match in %	GenBank Accession No.	Medium
	Shape	Margin	Elevation	Surface	Colour	Gram stain					
SE1	Circular	Entire	Raised	Smooth	White	+ve	*Staphylococcus equorum*	Shell	100%	JX315320	[a]BPA
SE2	Circular	Entire	Raised	Smooth	White	+ve	*Staphylococcus equorum*	Shell	98%	LN774385	BPA
SE3	Circular	Entire	Raised	Smooth	White	+ve	*Staphylococcus equorum*	Shell	99%	KJ920933	BPA
SE4	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus caprae*	Shell	100%	HG421011	BPA
SE5	Circular	Entire	Raised	Smooth	White	+ve	*Staphylococcus equorum*	Shell	100%	LN774571	BPA
SE6	Circular	Entire	Raised	Smooth	White	+ve	*Staphylococcus equorum*	Shell	99%	KR012324	BPA
SE7	Circular	Entire	Raised	Smooth	Cream	+ve	*Clostridium perfringens*	Shell	99%	KP944158	[b]RCA
SE8	Circular	Entire	Flat	Smooth	White	+ve	*Enterococcus faecium*	Shell	99%	KC715828	RCA
SE9	Circular	Entire	Raised	Smooth	Cream	+ve	*Clostridium perfringens*	Shell	99%	KP944158	RCA
SE10	Circular	Entire	Raised	Smooth	Cream	+ve	*Staphylococcus haemolyticus*	Shell	99%	KF318857	[c]LSA

[a]Baird Parker agar; [b]Reinforced Clostridial agar; [c]*Listeria* selective agar

The average TVCs isolated on RCA plates were estimated to be 9 x 10^2 CFU/eggshell. Three isolates were analysed by sequencing part of the 16S rRNA gene, and the result showed that two isolates, SE7 and SE9 had identity of 99% to *Clostridium perfringens*, while the isolate SE8 had identity of 99% to *Enterococcus faecium*. *C. perfringens* appeared on RCA with a glossy colony that had entire margin and a moderate size, whereas *E. faecium* had a small size colony with circular shape and entire margin (Figure 2.7).

Clostridium perfringens and *Enterococcus faecium* are components of the normal intestinal flora of poultry and humans. They are both significantly pathogenic and can cause life threatening infections and other illnesses (Sakurai *et al*. 2004). *E. faecium* was previously isolated from intestine of poultry, and it can cause life threating infection in human, particularly in the nosocomial environment, in which a naturally high level of antibiotic resistance is found in *E. faecium*. It has been also found to cause bacteraemia, endocarditis, urinary tract infections and meningitis.

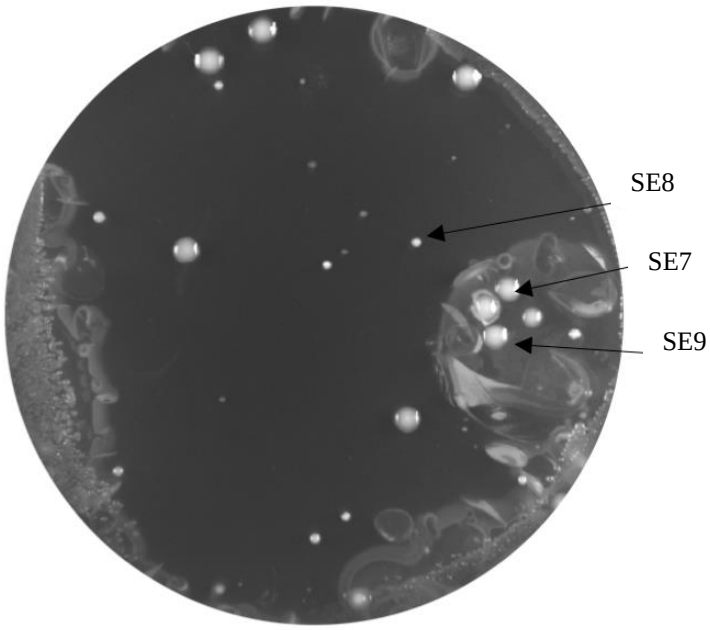

Figure 2.7 Bacteria isolated from the eggshell on Reinforced Clostridial agar at dilution of 10^{-1} and incubated anaerobically at 37 °C. The sequencing results showed that SE 7 and SE 9 were *Clostridium perfringens*; SE 8, *Enterococcus faecium*.

Of the 16 eggshell samples tested for the presence of clostridia, three egg samples showed bacterial growth, but two isolates out of three colonies tested were found to be *Clostridium*. Clostridia were not detected earlier by plating on PCA, because of the specific growth requirements of these organisms, specifically the need for anaerobic conditions.

Samples isolated from the eggshell and content and cultured on BGA, EMB and CCDA for targeting *Salmonella*, coliforms and *Campylobacter* showed no bacterial growth. In terms of presence of *Salmonella* in eggs, a study by Perales and Audicana (1989) showed that the prevalence of *Salmonella* from the eggshell was 1.1%, from a total of 372 eggshells tested. Another study by Musgrove *et al*. (2004) found the occurrence of *Salmonella* was 1.2 % from a total of 84 eggs tested. In terms of *Salmonella* from egg content, the prevalence can be variable depending on the sample size, sites within the egg that were tested and the technique used (De Reu *et al*., 2006a). The prevalence of *Salmonella* in egg content was estimated in different studies as 0.03% from a total of 46200 eggs and 0.77% from a total of 1304 eggs respectively (Boer and Wit 2000; De Reu *et al*. 2008).

There are no data found in publications that have estimated the prevalence of *Campylobacter* from table eggs. The results obtained in this study suggest no *Salmonella*, *Campylobacter*, *E.coli* or *Listeria* bacteria were identified.

2.5 Discussion and conclusion

Table eggs are considered as a main cheap protein source that is consumed by people around the world (De Reu *et al*., 2008; Humphrey *et al*., 1989; Adesiyun *et al*., 2005). Also, many processed foods nowadays may contain eggs as one of the main ingredients. Therefore, it is important to ensure that the eggs consumed are free of pathogens that might lead to serious illnesses. A number of studies have focused on the quality of table eggs using the conventional method of isolating bacteria on nutrient media and identifying the isolates by studying their biochemical characteristics (Elliott, 1954; Alvarez-Fernández *et al.*, 2012; De Reu *et al*., 2008). In the present study, analysis was extended by using the 16S rRNA gene sequence as a tool for identification. The advantage of this approach is the ability to define the bacterial species accurately. The aim of the study was to assess the microbial diversity of table eggs, and to determine the quality of eggs sold in the market.

The average of TVCs from the free range eggshells obtained in this study was 5.7 x 10^5 CFU/eggshell, followed by organic free range with an average TVCs of 2.9 x 10^5 CFU/eggshell and by comparison with 2 x 10^5 CFU/eggshell for the caged eggs, in agreement with studies performed by Knape *et al*. (1999) and De Reu *et al*. (2009). However, A study by Alvarez-Fernández *et al*. (2012) showed lower microbial loads from the eggshell of organic free range accounted for 1.7 x 10^2 CFU/cm^2, followed by free range with an average of 1.5 x 10^2 CFU/cm^2 and 9.1 x10^1 CFU/cm^2 for caged eggs. The calculation of the TVCs per cm^2 which represents a small area of the egg compared with immersing the whole egg in diluent buffer and calculating the TVCs/ eggshell. Therefore, many factors can affect the TVCs, including treatment of eggs used when isolating bacteria. For example, some studies have used swab sampling.

Moreover, different regulations in various studies for handling eggs could be a reason behind the variation of TVCs isolated from the eggshell, for instance in the European union, washing class A eggs is banned, but they are routinely washed in the United States, Japan, Canada and Australia to reduce shell contamination, therefore, reducing egg spoilage (Hutchison *et al.*, 2004). In a study by Hannah *et al.* (2011) a comparison of TVCs from unwashed and washed eggshells in which the results showed washing significantly reduced the TVCs to 2.2 $\log_{10}$ CFU/ml. In addition, egg samples from the

market shelves tend to have higher microbial load than samples collected directly from the farm, since the eggs have gone through different handling processes, including collection, size sorting, and packaging (Cader *et al*., 2014). Thus, these handling processes might increase the TVCs of the eggshell. There was a variation in the TVCs of eggshell from different housing systems, however, the variation was not significant ($P \geq 0.141$) by performing the one-way ANOVA test between the variables. One reason that could result in free range eggs having higher TVCs than caged eggs is that the free range eggs are laid in the environment rather than in cleaned or disinfected cages. Therefore, the chance that the free range eggs will be contaminated with environmental microorganisms is higher than for the caged eggs.

Previous studies claimed that increasing the TVCs of eggshell might lead to increased TVCs in egg content (Sauter and Petersen, 1974; Messens *et al*., 2006). The reason might be because of deposition of the bacteria on the eggshell which might facilitate the penetration of bacteria through the eggshell to contaminate the egg content. *Salmonella*, *Campylobacter*, *E.coli* and other enteric pathogens are commonly associated with poultry and table eggs (Humphrey, 1994; De Reu *et al*, 2008). However, the results of this study revealed that none of these enteric bacteria were identified, but other bacteria including *Clostridium perfringens* and *Enterococcus faecium* were isolated from the eggshell, and they may still pose a threat to consumers. A wide range of different bacteria were isolated from the eggs. The most frequently occurring strains among 47 isolates identified were found to belong to the genus *Staphylococcus*, and this finding in agreement with the study performed by Stepień-Pyśniak *et al.*, (2009) suggested that *Staphylococcus* species were the major contaminants from table eggs. Also, *Micrococcus* was found to be the second most common contaminant, agreeing with the results obtained by Chaemsanit *et al.* (2015).

After studying microbial community of table eggs using the culturing technique for bacterial isolation and using the 16S rRNA gene for identifying these isolates, it was then of interest to develop a methodology for identifying bacterial flora from table eggs without using the culturing technique in order to detect VBNC bacteria that could be responsible for egg spoilage.

CHAPTER 3 : Identification of bacteria from table eggs using 16S rRNA gene cloning and sequencing

3.1 Introduction

Table eggs harbour a complex microbial community which plays a major role in determining the egg shelf life (Gram *et al.*, 2002). Despite the efforts that have been conducted for determining bacteria present in table eggs, there is still concern about relying on culture techniques, since this may provide an insufficient picture of the entire bacterial diversity. In other words, the presence of other bacteria that require particular nutrient requirements and other intrinsic factors for them to grow on a culture medium has not been established. Furthermore, bacteria can enter a viable but nonculturable (VBNC) state, in which they may not grow on normal culture media, but they are still alive and capable of renewed metabolic activity (Oliver, 2005). Therefore, conducting a direct analysis of the 16S rRNA gene for bacteria that are isolated from a mixed bacterial community may provide a more complete overview in organisms of table eggs. Thus, combining two bacterial identification methods by using the conventional culture approach and the sequencing approach may provide a more complete picture of the bacterial community that is found in table eggs.

This approach of molecular ecology offers a potential method for determining the whole diversity of prokaryotic taxa with no demands of using growth media or selective laboratory enrichment. Molecular genetic analysis of bacterial rDNA extracted from food samples is now routinely carried out in many laboratories worldwide, with the analysis performed without a requirement for culturing the organisms (Wintzingerode *et al*., 2006; Liesack and Stackebrandt, 1992). The standard protocol of the bacterial isolates analysis involves isolation of nucleic acids from a food sample, followed by PCR amplification using universal primers that target a specific region of the 16S rRNA genes (Sipos *et al.*, 2007). An attractive feature of using PCR is that it does not require high molecular weight DNA for successful amplification. However, obtaining nucleic acids suitable for PCR amplification is still challenging, since the presence of inhibitor components such as humic acids might impede the amplification process. Some protocols used for bacterial DNA extraction utilise a long treatment and organic solvent purification for removing inhibitory matters (Harnpicharnchai *et al.*, 2007; Sepp *et al.*, 1994).

Previous studies have attempted to extract bacterial DNA from environmental samples, such as soil, water and stones (Zhou *et al.*, 1996; Leff *et al.*, 1995; Mudariki *et al.*, 2013). Thus, extraction of bacterial DNA from the eggshell may be similar to the extraction from those environmental samples. On the other hand, extracting bacterial DNA from egg content is more challenging, because microbial cells may remain tightly attached to the egg proteins, and to other organic matter. To the best of our knowledge, no study has attempted to isolate bacterial DNA from both the eggshell and egg content.

Once the DNA has been isolated, PCR optimisation can result in an efficient reaction, in which microgram quantities may be produced from a single molecule of substrate DNA (Rychlik *et al.*, 1990). The optimisation requires optimising a number of variables, including annealing temperature (T_a), and magnesium chloride concentration (Innis and Gelfand, 1999). Another factor that may increase PCR yield is the amount of template DNA. Optimising a PCR reaction might improve the DNA yields obtained, and facilitate amplifying DNA molecules from a low amount of template DNA.

There are various types of protocols used by researchers for extracting bacterial DNA from environmental, clinical and food samples (Torsvik *et al.*,1990; Torsvik, 1980; Pinto *et al*., 2007; McOrist *et al*., 2002). Rapid boiling methods have been used extensively for isolating bacterial DNA, due to the fact that they are a fast, reliable and cheap way of DNA extraction (Lench, 1988; Omar *et al*., 2014; Sepp *et al*., 1994; Reischl *et al*., 2000; Holmes and Quigley, 1981). The procedure involves physical and mechanical forces to damage the bacterial cell wall and release the cell content. Other methods and kits are also available to be used, which involve chemical and mechanical treatments for extracting and purifying bacterial DNA. Some protocols are more time consuming, but still efficient in extracting particular samples. For example, samples that contain high amounts of proteins require a procedure that has a deproteinisation treatment, and use of chemical agents to remove excess of organic materials. Thus, it is important for successful DNA extraction to select the proper extraction procedure based on the nature of the treated sample.

3.2 Objectives

The first objective of this study was to develop a methodology for extracting bacteria DNA directly from eggshell and egg content. The second objective of this study was therefore to identify bacteria present on eggshell and in egg content by sequencing the 16S rRNA gene directly without culturing or enriching the organisms.

3.3 Materials and methods

The first part of the experiment was focused on developing methodologies for extracting bacterial DNA from the eggshell rinse and from egg content homogenate, in order to analyse and identify those bacterial DNA by using the cloning approach.

3.3.1 Bacterial strains used for developing DNA extraction methods

Two bacterial strains were tested for PCR optimisation and DNA extraction- *Escherichia coli* (CP000946) and *Staphylococcus equorum*. These strains were selected since *S. equorum* (HG941668) is a Gram-positive bacterium and they were found in earlier work to be major contaminants of eggs, and *E. coli* is a Gram-negative bacterium and was involved in several outbreaks associated with eggs, and also was found to facilitate penetration of *Staphylococcus* (Al-Natour *et al.*, 2012). The bacterial strains were cultured in 10 ml LB broth and incubated at 37 °C for 24 h. The culture was then serially diluted up to 10^{-6} by successively inoculating 1 ml of bacterial culture into 9 ml 1x phosphate buffer saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.0), followed by plating 100 µl of each dilution on plate count agar, and incubating at 37 °C for 24 h. The viable counts were calculated to be 10^8 CFU/ml for *S. equorum*, whereas *E. coli* was 10^7 CFU/ml. 1 ml of each dilution was centrifuged for 3 minutes at x 13000 rpm. The supernatant was discarded and the pellet placed at -20 °C until it was required.

The 16S rDNA primers used in this study were 9F (5′-GAGTTTGATCCTGGCTCAG-3′; position 9-27, *Escherichia coli* 16S rRNA numbering) and 536R (5′-GTATTACCGCGGCTGCTG-3′; position 536-519). These primers were previously utilised by Kim *et al*. (2004) for detection of bacterial isolate from water samples. It has been suggested that for most bacterial isolates the initial 500-bp sequence provides adequate differentiation for identification (Clarridge, 2004). PCR was performed in a total volume of 50 µl. The reaction contained 25 µl of 2x BioMix buffer (Bioline), 1 µl forward primer (10 µM 9F RNA), 1 µl reverse primer (10 µM 536R RNA) and 2 µl DNA template (concentration 93 ng/µl for *S. equorum* and 64 ng/µl for *E. coli*). The PCR mixture was heated at 95 °C in a thermal cycler for 4 min, followed by 30 reaction cycles of 95 °C for 30 seconds, followed by annealing step at 54 °C for 30 seconds, and elongating step at 72 for 30 seconds; followed by final extension at 72 °C for 7 minutes. Annealing temperature

was varied as part of annealing temperature optimisation. The primer melting temperature was calculated to be 58 °C using the formula T_m = (G+C) x 4 + (A+T) x 2, and 6 T_a temperatures were selected based on the calculations of T_m.

3.3.2 A comparison of 5 different nucleic acids extraction procedures

To extract the cell lysate from bacterial cells that contain nucleic acid, 5 extraction methods were compared. Modified boiling extraction (MBE), Chelex-100 extraction (CE), Calcium Carbonate extraction (CCE), Phenol-chloroform extraction (PCE), and GentraPuregene®kit (GPK)(Qiagen). The MBE involved incubating bacterial pellet in 20 µl ddH_2O at 85°C for 20 minutes with occasional vortexing. The modification lies in extending the time to 20 minutes and decreasing temperature from boiling degree 100°C to 85°C. The sample was then placed on ice for 5 minutes and centrifuged at high speed, 13000 rpm for 3 minutes. CE was a similar method to MBE using 20 µl of 5 % Chelex-100 instead of 20 µl ddH_2O that was used in the previous method. The sample was then incubated for 20 minutes at 85°C, then placed on ice and centrifuged for 3 minutes. Also, the CCE procedure was similar to CE, but replaced Chelex-100 with 20 µl of 5% $CaCO_3$. In PCE a method described by Ausubel *et al*. (1994) was followed, which involved resuspending bacterial pellets in 567 µl TE buffer. 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added. The aliquot was mixed thoroughly and incubated for 1 h at 37 °C. 100 µl of 5M NaCL was added and vortexed. Then, 80 µl of CTAB/NaCl (0.7M NaCl, 10% CTAB) solution was added, mixed and incubated 10 minutes at 65 °C. The sample was extracted with 700 µl of chloroform/isoamyl alcohol and centrifuged for 5 minutes, the aliquot was discarded and the pellet was resuspended in 20 µl ddH_2O . For the last method GPK, the protocol provided for extracting bacterial DNA was followed, and finally DNA was visualised on a gel to compare DNA concentration of each method used.

3.3.3 Isolation of bacterial genomic DNA from eggshell rinse

This method was intended to extract bacterial genomic DNA from the eggshell rinse, without enriching or culturing the bacterial cells in a specific medium. The method involved placing an egg in a sterile bag containing 10 ml 1x phosphate-buffered saline (PBS), and rubbing thoroughly for 20 seconds to detach the bacteria from the eggshell.

The eggshell rinse was transferred to a sterile 15-ml Falcon tube, and centrifuged at 8000 rpm for 10 min. The supernatant was discarded and the pellet resuspended in 1ml 1x PBS, and the aliquot was transferred to a sterile Eppendorf tube, and then was centrifuged at 13,000 rpm for 3 minutes. The supernatant was discarded and the pellet was subjected to DNA extraction. MBE was used to extract DNA. The pellet was resuspended in 20 µl sterile nuclease-free water, and then the sample was incubated at 85 °C for 20 minutes. The mixture then was placed on ice for 5 minutes and was centrifuged at x 13000 rpm for 3 minutes. This procedure was applied to 6 eggshells to ensure the effectiveness of the technique.

3.3.4 Isolation of microbial DNA from egg content homogenate

A preliminary treatment to disinfect the eggshell was performed by immersing the eggshell in 70 % EtOH for 10 seconds, and then the egg was flamed for 2 seconds. The egg was cracked aseptically, and 25 g of egg content evacuated into a plastic bag containing 9 volumes 1x PBS, then was homogenised for 30 seconds in a stomacher® 400 to mix the sample with diluent buffer. The homogenate then was centrifuged in a Beckman Coulter centrifuge at 10,000 rpm for 20 minutes. The supernatant was removed and the pellet placed in a sterile plastic bag containing 5 ml ¼ strength Ringer's solution and homogenized in the stomacher® 400 for 2 minutes. 1.5 ml of the homogenate was used for total genomic DNA extraction following a protocol described in the PowerFood®Microbial DNA isolation kit (MOI*BIO*), the kit is designed to recover microbial DNA from food samples. The boiling extraction method was not suitable to extract bacterial DNA from the egg content, since the high temperature leads to solidification of the egg content protein in the sample, therefore complicating the extraction process. A work flow showing treatment stages is shown in Figure 3.1.

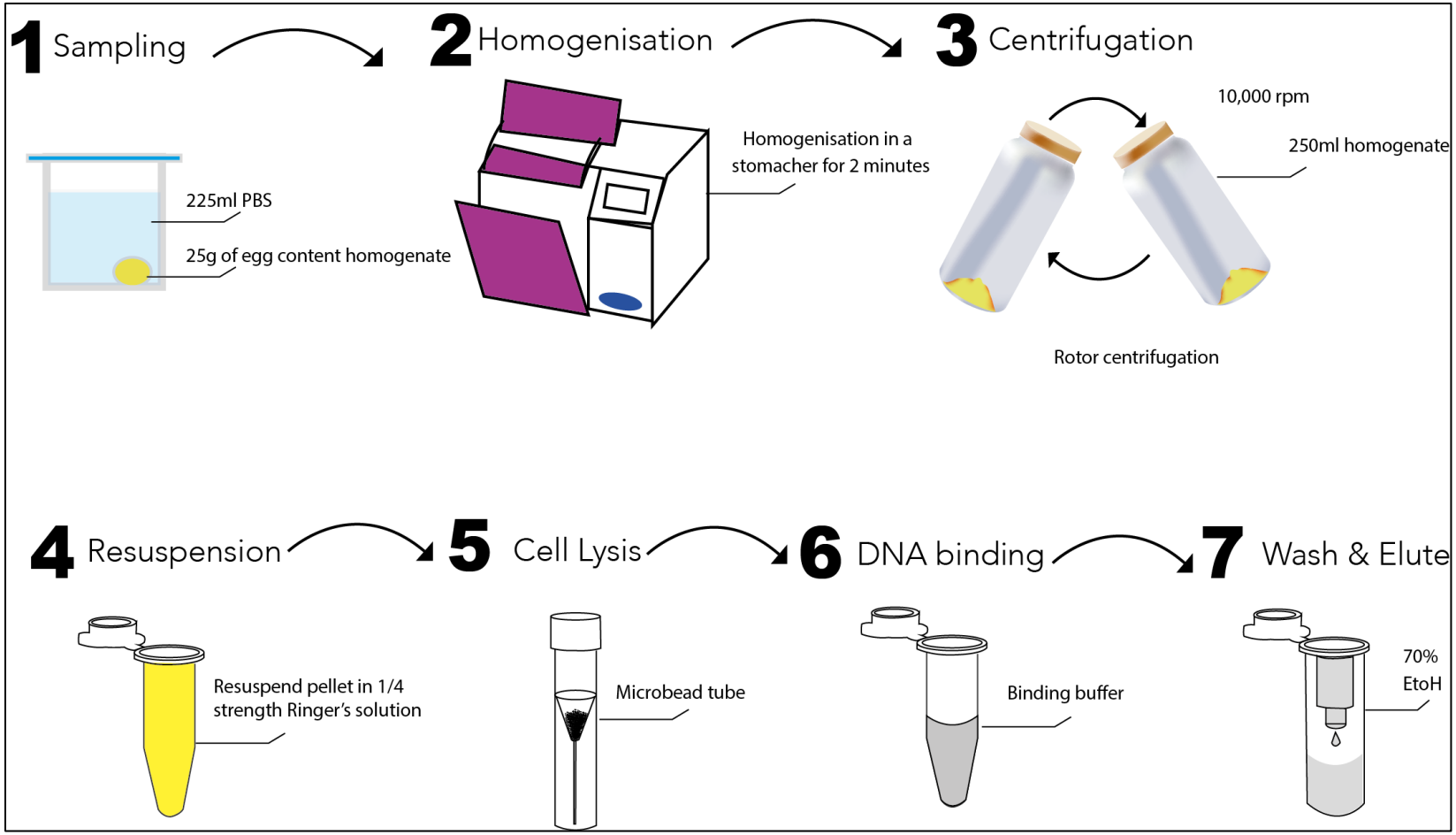

Figure 3.1 Work flow of extracting bacterial genomic DNA from egg content. The first step of the extraction was mixing 25 g of egg content with 225 ml 1x PBS, then was homogenised in a Stomacher for 30 seconds, and was centrifuged at 10,000 rpm for 20 minutes. The supernatant was discarded and the pellet was placed in a sterile plastic bag and resuspended with sterilised 5 ml 1x PBS, then homogenised for 2 minutes. Afterwards, 1.5 ml of the homogenate was subjected to DNA extraction using PowerFood®Microbial kit (MOBIO). The instructions provided in the kit were followed.

3.3.5 Polymerase chain reaction

The bacterial cell lysate from the eggshell that contained template DNA were amplified using PCR. The PCR reaction mix contained 25 µl Easy-A high fidelity master mix (Agilent), 1 µl forward primer (10 µM 9F RNA), 1 µl reverse primer (10 µM 536R RNA), 2 µl of cell lysate recovered from the eggshell including DNA template and 21 µl nuclease-free water, and the reaction was run using the same cycles as described in section 2.3.8. In terms of egg content, a reaction mix of 25 µl Easy-A high fidelity master mix (Agilent), 1 µl forward primer (10 µM 9F RNA), 1 µl reverse primer (10 µM 536R RNA), 2 µl purified microbial DNA extracted from egg content homogenate and 21 µl nuclease-free water and the reaction was run using the same cycles used for the eggshell procedure.

3.3.6 Detection of the PCR products

The PCR products were run on a 1% (w/v) agarose gel to detect the DNA amplification product. The gel was prepared as described previously in section 2.3.9.

3.3.7 Cloning of the 16S rRNA Gene

In order to identify mixed bacterial communities in a sample using the 16S rRNA gene, and without culturing them in a growth medium, bacterial genomic DNA should be extracted and amplified by PCR. However, extracting genomic DNA from a mixed bacterial population will obviously generate bulk DNA fragments extracted from different organisms, which makes it difficult for sequencing. Therefore, DNA fragments were separated by cloning. The experimental procedure is summarised in Figure 3.2. The cloning strategy used in this study was that of the TA cloning system, using the Strata Clone (Agilent cloning kit). This kit was selected as it is a reliable and rapid cloning kit, which is designed for efficient cloning of *Taq* polymerase-generated fragments that contain a 5'-A overhang. The Strata Clone vector used in this procedure has 3'-T overhangs to create efficient cloning of *Taq* polymerase-generated fragments. This strategy promotes easy screening for clones using the blue/white screening technique. In the ligation step, PCR amplified linear DNA fragments were ligated with Strata Clone vector (pSC-A-amp/kan).

The ligation mixture was prepared from 3 µl Strata Clone cloning buffer, 2 µl of the PCR product (5-50 ng), and 1 µl Strata Clone vector mix amp/kan. The mixture was incubated at room temperature for 5 minutes and then was placed on ice until further processing.

3.3.8 Transformation of competent cells

Transformation was carried out by following the protocol of the Strata Clone Cloning kit. A tube of Strata Clone Solo Pack competent cells was placed on ice for each ligation reaction. 1 µl of the ligation mixture was added to the competent cells, and mixed by tapping the tube gently. The transformation mixture was kept on ice for 20 minutes, and then incubated at 42 °C for 45 seconds to perform heat-shock. Afterwards, the transformation was placed on ice for 2 minutes. 250 µl of pre-warmed LB medium was added to the transformation reaction mixture, followed by incubation at 37 °C with horizontal shaking for one hour. The culture was plated on LB agar containing ampicillin 50 µg/ml and 40 µg/ml X-gal (for blue-white screening), and incubated at 37 °C for 24 hrs. The X-gal allowed the screening for inactivated β – galactosidase activity. Therefore, clones that carry an inserted gene will produce white colonies which can be discriminated from blue colonies, which are presumed to contain non-recombinant plasmids.

3.3.9 Screening and analysis of clones

The clones were examined for white colonies that should contain a 16S rDNA gene. The clones were then picked and sub-cultured on a LB agar plate containing 50 µg/ml ampicillin for plasmid extractions.

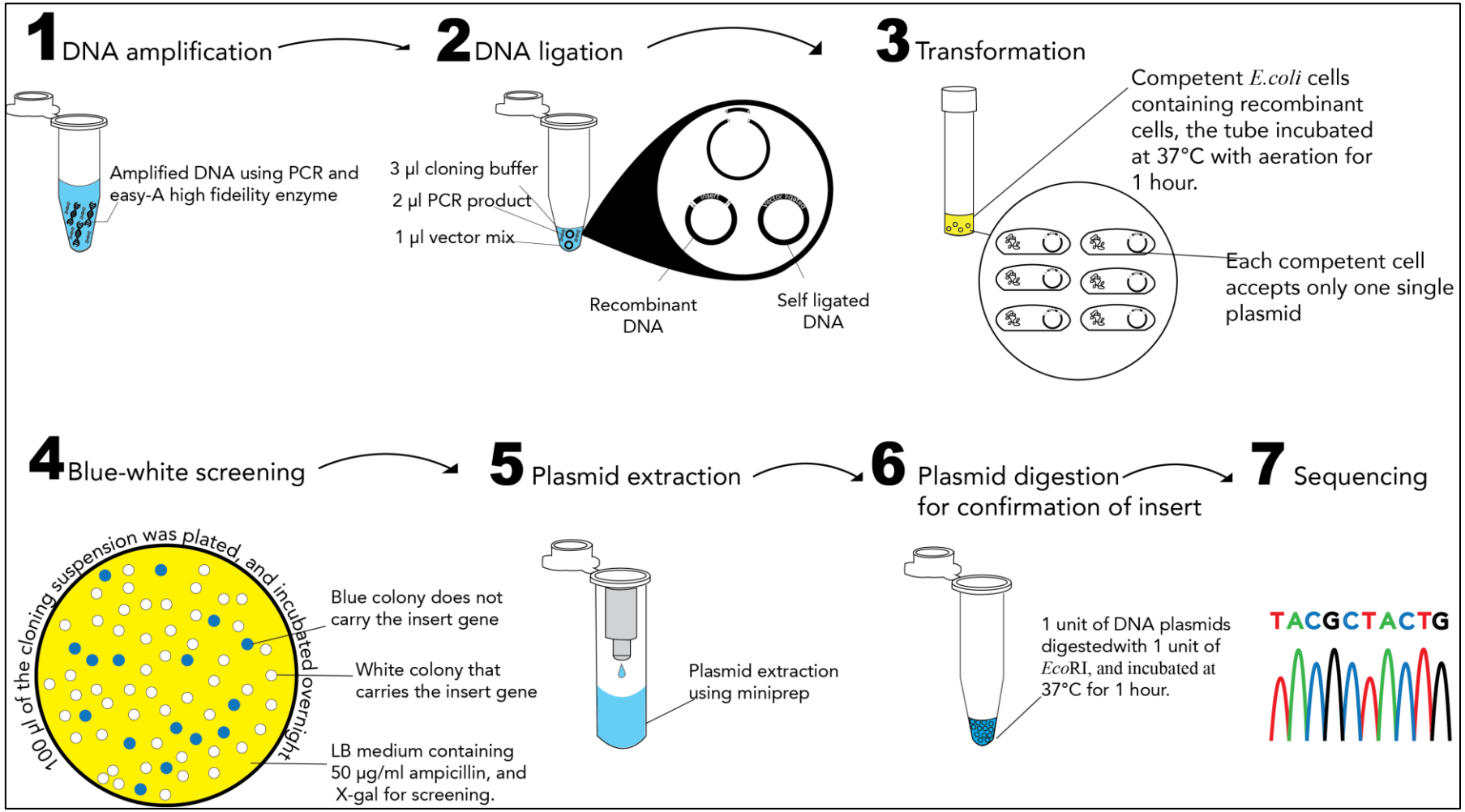

Figure 3.2 Direct analysis of bacterial diversity in table eggs by using the cloning approach. The figure shows the treatments steps performed to characterize the mixed bacterial community including the insert confirmatory test.

3.3.10 Preparation of plasmids by miniprep procedure

Plasmids were purified using the Thermo-Scientific GeneJet™ Plasmid Miniprep Kit #K0502. Cloned cells were inoculated into 5 ml of LB broth containing 100 µg/ml ampicillin. The culture was incubated at 37 °C with agitation overnight. 5 ml of the culture was centrifuged at 8000 rpm for 15 minutes to pellet the cells. The supernatant was discarded, and the pellet was resuspended in 250 µl of the resuspension solution by vortexing. The aliquot was transferred to a clean Eppendorf tube, and 250 µl of the lysis solution was added, and mixed thoroughly by inverting the tube 6 times. 350 µL of the neutralization solution was added, and mixed thoroughly by inverting the tube. Centrifugation at 13,000 rpm for 5 minutes was performed to pellet cell debris and chromosomal DNA. The supernatant was transferred to a spin column containing silica resin supplied in the kit by decanting to avoid disturbing the pelleted debris, and centrifuged for 1 minute. 500 µL of the wash solution (diluted with ethanol) was added to the column and centrifuged for 1 minute. The flow through was discarded and a second wash was performed followed by centrifugation for 1 minute. Another centrifugation for 1 minute was conducted to remove any residual ethanol. The GeneJET spin column was finally placed into a sterilise 1.5 ml microcentrifuge tube and 50 µL of elution buffer was added. The tube was left to stand for 2 minutes and centrifuged at 13,000 rpm for 2 minutes. The purified plasmid DNA was stored at -20 °C until needed for further analysis.

3.3.11 Plasmid digestion

In order to confirm that the recombinant plasmid contained targeted insert, plasmid digestion was performed using *Eco*RI enzyme. A reaction mix of 2 µl plasmid DNA (500 ng), 2 µl *Eco*RI buffer, 1 µl *Eco*RI digestion enzyme, and 15 µl ddH_2O was made. The mixture was then incubated at 37 °C for an hour. The digestion enzyme was inactivated by incubating the mixture at 75 °C for 15 minutes. A visual screening of the digested fragments was performed by 1% agarose gel electrophoresis, staining the DNA with ethidium bromide.

3.3.12 Plasmid sequencing

For identifying the insert in each plasmid, sequencing of the cloned 16S rRNA gene was required. The samples were sequenced in both forward and reverse direction, to reduce base-calling errors and PCR ambiguities. In 0.2 ml MicroAmp tubes (Applied

Biosystems), 1 µl of plasmid DNA was mixed with 1 µl forward or 1 µl reverse primer (5 pmol/µl) and 4 µl ddH_2O. 200 samples from the eggshell and egg content were prepared for gene analysis. 91 samples were sent the GenePool group in University of Edinburgh (http://genepool.bio.ed.ac.uk). The remaining samples were kept in -20 °C until they were sent for gene sequencing analysis.

Sequencing Chromatograms were visualised using 4 Peaks version 1.8, and flanking plasmid sequences were removed. Then, the forward sequence was aligned with reverse sequence using the align tool in the NCBI website; (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=blast2seq&LINK_LOC=align2seq.)

After all the sequences were aligned, they were analysed using the tool BLASTN from the NCBI website, to determine phylogenetic similarities with other sequences available in the database. Phylogenetic analysis was carried out on the basis of 16S rRNA sequences. For phylogenetic analysis of table egg clones, the sequences were aligned and trees constructed by applying a neighbour-joining method. The tree was constructed using MEGA 6 software.

3.4 Results

In previous works in this study, the bacterial flora from table eggs was observed using the culturing technique, in which the bacterial isolates were identified by using the 16S rRNA gene sequences. Then, it was of interest to try to extend the observation by identifying bacterial flora that are viable but non-culturable due to the growth requirements that are needed.

3.4.1 Optimisation of DNA extraction

For the purpose of studying VBNC bacterial communities from table eggs, extracting the genomic DNA directly from bacterial cells that are present on the eggshell and in egg content is required. Therefore, an efficient method for direct extraction of bacterial DNA was developed. A comparison of 5 DNA extraction methods was performed to isolate genomic DNA from two bacterial strains that were serially diluted and prepared for DNA extraction as explained in the methodology section 3.3.2.

In order to determine the number of bacterial cells, which are required to generate PCR amplicons that can be seen as a clear band on a gel, it was necessary to prepare serial dilutions of the tested bacteria, and to quantify total viable counts. TVCs were calculated to be for the main inoculum ~ 10^8 and ~ 10^7 cells for *S. equorum* and *E. coli* respectively. Therefore, 6 samples that contain known number of bacterial cells of *S. equorum* ~10^7 to 10^2 cells/ml, and another 6 samples of *E. coli*, ~10^6 to 10^1 cells/ml were prepared for each extraction method.

The MBE method revealed its efficiency to extract bacterial DNA and provide a visible PCR amplicon from a low concentration of *S. equorum* cells ~ 10^4 cells, followed by CCE, which had nearly the same effectiveness, but with lower PCR product concentration that can be distinguished as shown in Figure 3.4. The GPK and PCE methods provided PCR products that could be seen on a gel from concentrations of ~ 10^7 and 10^6 cells, but PCR bands were invisible on the gel when reducing concentration of bacterial cells in the sample. In terms of CE, very low DNA concentrations were observed from all samples extracted, which indicates unsuitability of this method for extracting DNA from *S. equorum*. All extraction methods were unable to provide a visible PCR band that can be

seen on a gel from an amount of ~ 10^3 cells. The size of DNA bands was in agreement with the expected size 590 bp.

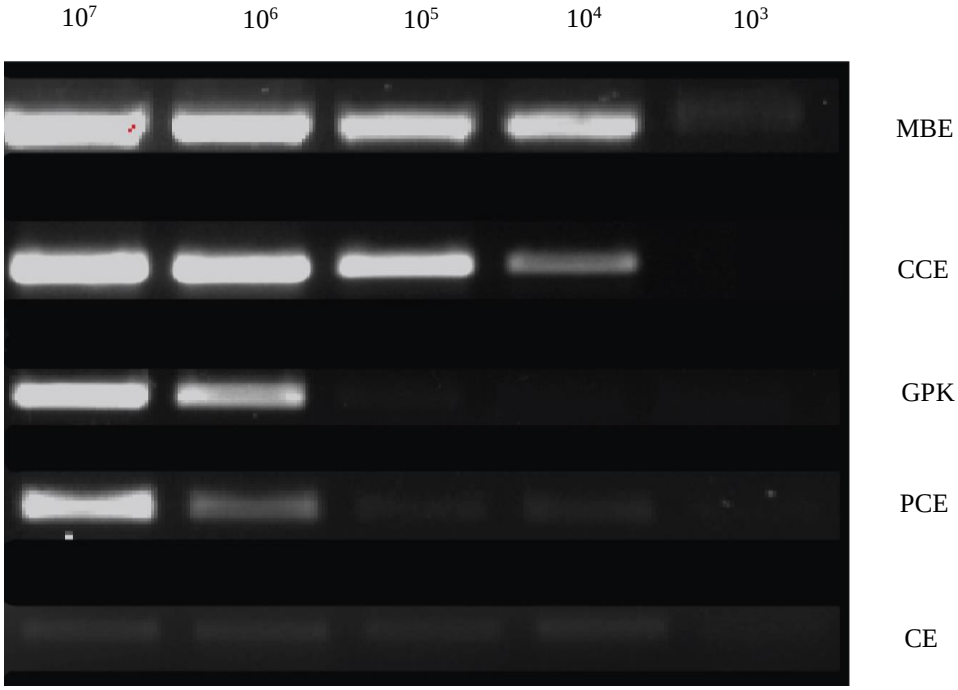

Figure 3.3 Agarose gel electrophoresis of PCR amplicons showing sensitivity of 5 different DNA extraction methods. DNA was extracted and amplified from *Staphylococcus equorum*. MBE, modified boiling extraction; CCE, calcium carbonate extraction; GPK, Gentra Puregene kit; PCE, phenol-chloroform extraction; CE, Chelex-100 extraction.

Similar results were obtained using the same methods to extract DNA from *E .coli.* DNA amplification was successfully obtained from 10^4 cells of *E.coli* bacterial cells using either MBE or CCE. As can be seen from Figure 3.4, the CE method for the extraction of *E. coli* cells has resulted in an intense PCR band from a concentration of 10^6 cells, whereas no PCR bands were observed using the same method for the extraction of *S. equorum* cells. However, no PCR bands were observed when reducing the amount of *E. coli* cells to 10^5 cells/ml, which clearly indicates that using CE method is not effective for the extraction of *E. coli* DNA from amount of cells lower than 10^6 cells.

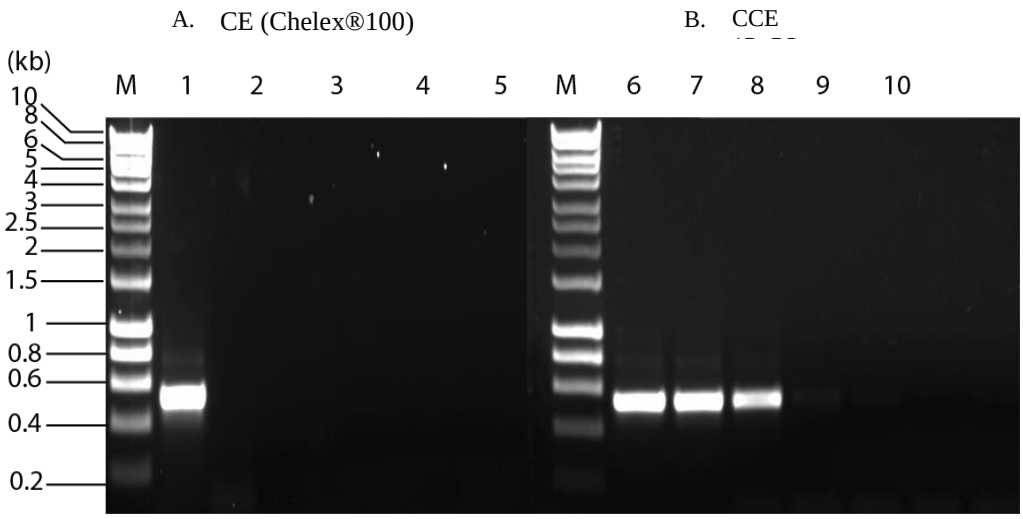

Figure 3.4 Agarose gel (A) electrophoresis showing PCR products from *E. coli* DNA using CE (Chelex®100); lane: M, Hyper Ladder I (Bioline); lane: 1, PCR DNA band produced from an amount of ~ 10^6 cells *E. coli* bacterial cells; lane: 2-5 inoculated with amplified PCR products by using a Chelex extraction method but no PCR DNA bands were detected. Agarose (B) using CCE method lane: 6, PCR product of amplified DNA produced from amount of 10^6 cells of *E.coli*; lane: 7, PCR band of amplified from an amount 10^5 cells; gel (B) lane : 8, 10^4 cells. Lane 9 and 10, no DNA bands were observed from 10^3 and 10^2 cells/ml respectively.

3.4.2 Direct isolation of bacterial DNA from eggshell rinse

The comparison of different DNA extraction methods has provided about the procedures needed to extract bacterial DNA. It is revealed that the minimum number of bacterial cells required to obtain PCR products that can be clearly visualised by gel electrophoresis was 10^4 cells using the MBE. Since, the average TVCs from eggshells was found to be ~10^5 CFU/eggshell, therefore, it should be practically possible to obtain PCR products suitable for cloning from that amount of bacterial cells. Accordingly, bacteria from eggshell were removed in a sterile plastic bag containing 10 ml 1x PBS as described in the methodology section 2.3.3. The eggshell wash was then centrifuged at high speed to pellet bacterial cells and the supernatant was discarded. This step ensured that there was a sufficient number of bacterial cells which could be treated for DNA extraction. DNA was extracted directly from the egg shell rinse using the MBE method, and was amplified with the 16S rDNA universal primers (Figure 3.5). The gel shows PCR products derived from bacterial DNA extracted directly from 6 different organic free range eggshells, and they were clearly observed. The size of the DNA bands obtained were in agreement with the

expected size of about 590 bp. The PCR product of sample number 3 was selected for the cloning step.

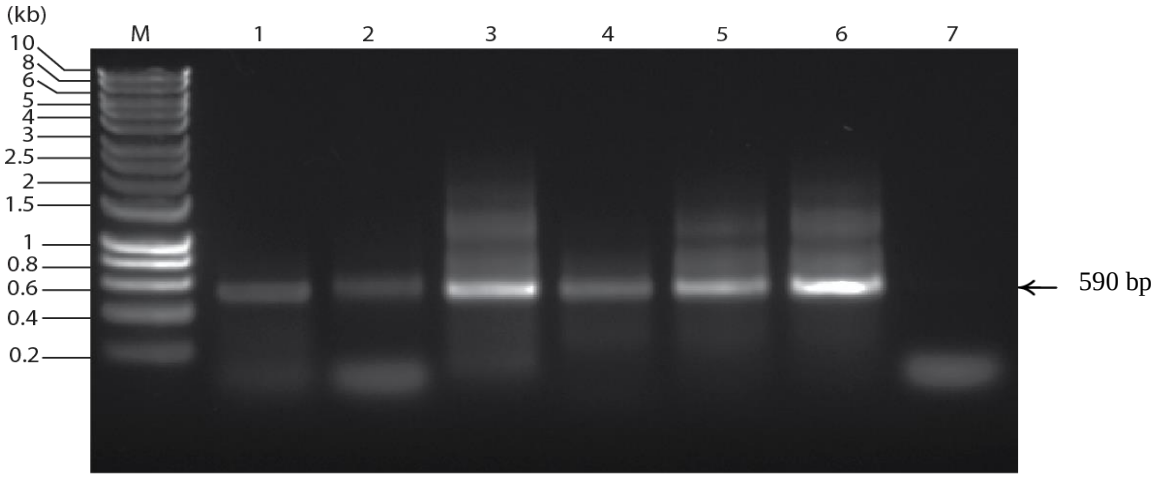

Figure 3.5 Agarose gel electrophoresis showing amplified DNA fragments derived from DNA extracted directly from eggshells. Lane M: hyper ladder I (Bioline), lanes 1:6 DNA extracted and amplified from bacteria that were isolated from 6 different eggshells. Different PCR band intensities were observed. lane 7, a negative control was performed without template nucleic acids.

3.4.3 Amplification of bacterial DNA from egg content homogenate

Extracting bacterial DNA from egg content homogenate was more challenging since the egg content contains a high percentage of protein that can impede the DNA extraction process, and bacterial number from egg content is lower than bacteria from eggshell. The MBE method used for extracting bacterial DNA from the eggshell rinse was inappropriate for extraction of bacterial DNA from the egg content, because of the high temperature that is used as the main step, which would result in solidification of the sample. However, another extraction method was developed to extract bacterial DNA from egg content, in which the sample was prepared before extracting as explained in section 3.3.5. The PowerFood®Microbial DNA isolation kit (MOI*BIO*) was designed specifically for microbial DNA extraction from food samples and was used following the protocol provided by the company. DNA was successfully extracted directly from the egg content homogenate of different free range eggs, and amplified by PCR with generation of DNA fragments of the right size (Figure 3.6). The PCR product of the sample number 3 was selected to be cloned and analysed.

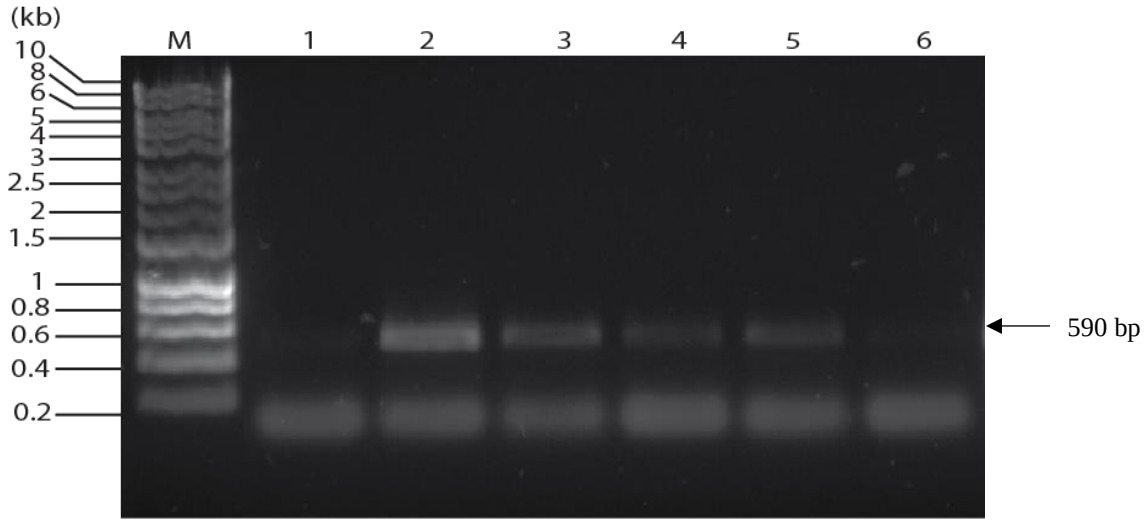

Figure 3.6 Agarose gel electrophoresis showing amplified DNA fragments derived from DNA extracted directly from egg content. Lane M: hyper ladder I (Bioline), lanes 1-6 DNA bands derived from 6 different egg content samples

The DNA bands that are shown in Figure 3.5 obtained from bacteria isolated from the eggshell had considerably higher intensity compared to bacterial DNA from egg content as shown in Figure 3.6. This is probably due to the fact that bacteria on eggshell are present in higher numbers than in the egg content, meaning that, higher bacterial DNA concentration could be derived from the eggshell. Presence of PCR inhibitors in a reaction are another factor that may inhibit amplification and reduce PCR product concentrations (Schrader *et al.*, 2012). Also, the primer-dimer can be clearly seen on the gel and this could be due to the high concentration of the primers in the PCR reaction or the low concentration of the DNA template.

As described in the introduction, using culturing techniques to study bacterial diversity of table eggs can provide inadequate information due to the occurrence of VBNC bacteria which require particular growth conditions. This part of the project involved direct analysis of bacteria from eggs without culturing them in growth media, by extracting bacterial DNA directly from the mixed bacterial community, and then amplifying it by PCR using high fidelity enzyme. The amplicons were ligated into a linear vector, and transferred into host competent cells for purification purposes.

3.5 Molecular cloning of 16S rRNA genes

After bacterial DNA was successfully isolated from both eggshell and egg content, and 16S rDNA was amplified using PCR. Strata Clone master mix that generated easy-A high-fidelity PCR product was used for the amplification. The aim of cloning the DNA fragments was to separate individual 16S rDNA sequences for analysis. All the clone sequences obtained in this study were generated from one organic free range egg.

3.6 DNA ligation

After the DNA fragments were successfully amplified, they were ligated into Strata Clone pSC-A-amp/kan vector provided in the Strata Clone kit (Figure 4.2). The recombinant DNA molecules were transformed into *Escherichia coli*, and plated on LB medium agar containing ampicillin and X-gal (for blue-white screening), and incubated overnight. The plate was examined for white colonies that should contain a 16S rDNA gene, so that they do not synthesise β-galactosidase to degrade X-gal. A few blue colonies were observed that represent vector only, whereas the majority of the colonies were white, and therefore considered as positive transformants that contain the target gene.

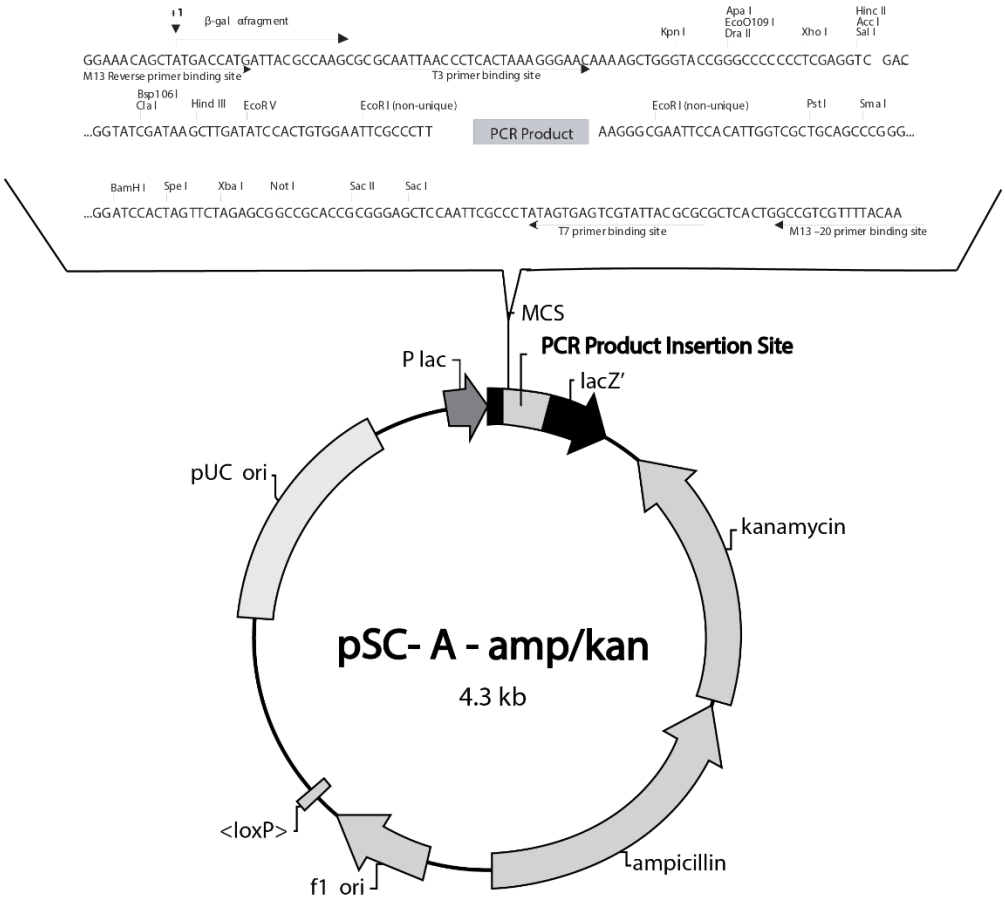

Figure 3.7 Vector map of pSC-A-amp/kan. The ligation site of the PCR product is shown above. Amplified 16S rDNA was cloned at this multiple cloning site (MCS) in the vector.

3.6.1 Plasmid isolation and restriction analysis

In order to confirm the presence of the cloned 16S rDNA gene, plasmids were extracted from cloned isolates as shown in Figure 3.7. Screening using PCR to amplify the inserted gene was excluded because the primer will potentially amplify the 16S rRNA gene present in the *E. coli* chromosome, providing a misleading result. Therefore, plasmids were digested with *Eco*RI as seen in Figure 3.8. The figure 3.9. revealed digested fragment with approximately the expected size of 570 bp representing the insert and 4.3kb (the original vector). The DNA fragments produced after digestion showed that they contain the correct size of the amplicon. Plasmids that were verified to contain an insert were subjected to DNA sequence analysis using 16S rDNA universal primers. A number of 91 samples were sequenced by the GenePool group in University of Edinburgh.

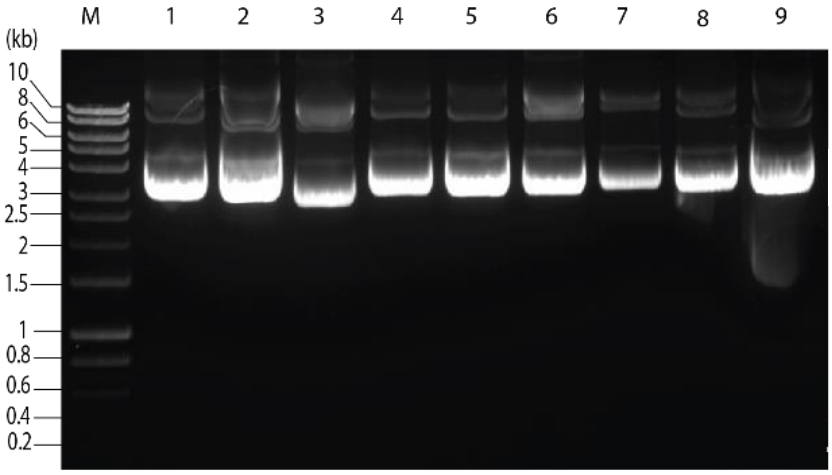

Figure 3.8 Agarose gel (1%) stained with ethidium bromide showing plasmids isolated following cloning of PCR-amplified 16S rDNA lane M: Hyper ladder I (Bioline), lane 1:9 plasmids isolated from individual colonies.

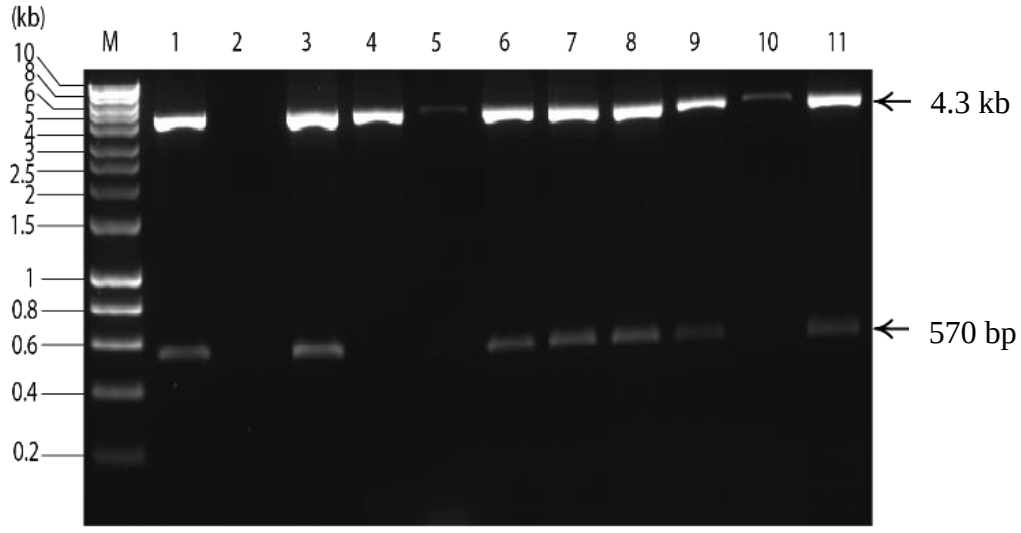

Figure 3.9 Recombinant plasmids constructed with pSC-A-amp/kan and digested with *Eco*RI. The cloned fragment can be seen in the figure with expected size 570bp M: Hyper ladder I (Bioline). Lane 1-11, plasmids isolated from individual colonies

3.6.2 Phylogenetic analysis on the basis of 16S rRNA sequences

Extracted plasmids were subjected to sequencing, in order to identify the source of the cloned inserts, and the sequence results were compiled using 4 peaks assembly software. Manual editing of the consensus sequence to exclude the PCR primer binding was performed and to remove any discrepancies between the two strands by evaluating the chromatographs. For identifying the closest match of the examined clones, analysis of 16S rDNA sequences was accomplished using BLASTN search from NCBI website and results are presented in Table 3.1.

The results showed that out of 80 cloned sequences from eggshell, there was a high occurrence of the genus *Psychrobacter* (44 cloned isolates, 55%), the species *Psychrobacter faecalis* represented (18 cloned isolates, 40%) of the total 44 *Psychrobacter* cloned isolates, followed by *Psychrobacter maritimus* (7 cloned isolates, 16%), *Psychrobacter pulmonis* (2 cloned isolates, 4.5 %), *Psychrobacter nivimaris* (2 cloned isolates, 4.5 %), *Psychrobacter frigidicola* (2 cloned isolates, 4.5 %), *Psychrobacter cibarius* (2 cloned isolates, 4.5 %), *Psychrobacter cryohalolentis* (2 cloned isolates, 2.2 %) and the rest were identified at the genus level *Psychrobacter* (11 cloned isolates, 25%).

From egg content the genus *Psychrobacter* occurred in 10 cloned isolates out of 11 clones analysed and the species *Psychrobacter faecalis* formed the high occurrence (9 cloned isolates, 90%). However, the sequences obtained for 14 clones within a species of *Psychrobacter faecalis* (CL88, CL16, CL25, CL56, CL94, CL90, CL87, CL5 and CL81) were found to be almost to Genbank accession number KX650120. The sequence identity values of these clones were equal or greater than 99%. The sequences obtained for the clones CL84, CL70, CL128, CL129, CL23 and CL58 were also almost identical and both shared the sequence identity value of 99%. These organisms should be considered to have a potential role in spoiling eggs, since that they can grow at low temperature (Dainty and Mackey, 1992).

The second genus that showed a high occurrence was *Acinetobacter* occurred in (15 cloned isolates, 19%) out of 80 isolates from the eggshell. 11 cloned isolates were found to be identical (CL21, CL15, CL19, CL26, CL28, CL29, CL35, CL36, CL39, CL44 and CL41). Followed by *Staphylococcus* (6 clones, 7.5%) and 2 of these clones were found

identical (CL55 and CL89). The rest of cloned isolates were identified at a frequency of less than 2.5 %, including *Clostridium, Actinobacter, Lactobacillus, Proteobacterium, Ralstonia* and *Olsenella.* However, none of these organisms were isolated from the egg content. It should be noted that the sequence analysed was a partial gene sequence of 527 nucleotides, rather than the full-length gene sequence, which in the case of the *E. coli* rRNA gene sequence is 1,450 nucleotides.

The population of sequences retrieved from both eggshell and egg content showed a high occurrence of sequences closely related to *Psychrobacter faecalis*. Interestingly, *Psychrobacter* species were not isolated previously using the culturing approach, and it could be a significant cause of egg spoilage, since it can grow at low temperature. Therefore, presence of this species in a high abundance makes it interesting to conduct more investigations on the occurrence of *Psychrobacter* in eggs. Identification analysis of the clone library showed that 89 % of the clones were matched with an assigned Genbank sequences and considered to be identified at species level if they had a total score match of equal or greater than 99%. 11 % of the cloned isolates were identified as uncultured bacterium clones.

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Table 3-1 Sequence results of the cloned inserts that were isolated from table egg bacteria

Isolates	Isolation parts	Identified clones	Identity %	Genbank Accession No.
CL6	Eggshell	*Psychrobacter faecalis*	99	JF710999
CL109	Egg content	*Psychrobacter faecalis*	100	KR051250
CL8	Eggshell	*Psychrobacter faecalis*	100	KR051250
CL88	Eggshell	*Psychrobacter faecalis*	99	KX650120
CL110	Egg content	*Psychrobacter faecalis*	100	KX650120
CL131	Egg content	*Psychrobacter faecalis*	100	KX650120
CL16	Eggshell	*Psychrobacter faecalis*	99	KX650120
CL25	Eggshell	*Psychrobacter faecalis*	99	KX650120
CL56	Eggshell	*Psychrobacter faecalis*	99	KX650120
CL94	Eggshell	*Psychrobacter faecalis*	99	KX650120
CL126	Egg content	*Psychrobacter faecalis*	99	KX650120
CL124	Egg content	*Psychrobacter faecalis*	99	KX650120
CL127	Egg content	*Psychrobacter faecalis*	100	KX650120
CL90	Eggshell	*Psychrobacter faecalis*	99	KX650120
CL87	Eggshell	*Psychrobacter faecalis*	99	KX650120
CL5	Eggshell	*Psychrobacter faecalis*	99	KX650120
CL81	Eggshell	*Psychrobacter faecalis*	99	KX650120
CL40	Eggshell	*Psychrobacter faecalis*	99	KT767856
CL84	Eggshell	*Psychrobacter faecalis*	99	KU364016
CL70	Eggshell	*Psychrobacter faecalis*	99	KU364016
CL128	Egg content	*Psychrobacter faecalis*	99	KU364016
CL129	Egg content	*Psychrobacter faecalis*	99	KU364016
CL23	Eggshell	*Psychrobacter faecalis*	99	KU364016
CL58	Eggshell	*Psychrobacter faecalis*	99	KU364016
CL130	Egg content	*Psychrobacter faecalis*	99	HQ698577
CL53	Eggshell	*Psychrobacter faecalis*	99	KX650144
CL86	Eggshell	*Psychrobacter faecalis*	99	KX650119
CL42	Eggshell	*Psychrobacter maritimus*	100	EU000245
CL17	Egg shell	*Psychrobacter maritimus*	99	EU000245
CL34	Eggshell	*Psychrobacter maritimus*	99	KJ939482
CL27	Eggshell	*Psychrobacter maritimus*	99	KJ939482
CL65	Eggshell	*Psychrobacter maritimus*	99	KJ939482
CL43	Eggshell	*Psychrobacter maritimus*	99	KJ939482
CL45	Eggshell	*Psychrobacter maritimus*	99	KJ939482
CL85	Eggshell	*Psychrobacter* sp.	99	JX196614
CL7	Eggshell	*Psychrobacter* sp.	100	KY406022
CL83	Eggshell	*Psychrobacter* sp.	99	KR029271
CL66	Eggshell	*Psychrobacter* sp.	99	KR029271
CL92	Eggshell	*Psychrobacter* sp	100	KR029400
CL125	Egg content	*Psychrobacter* sp.	99	KR029400
CL9	Eggshell	*Psychrobacter* sp.	96	KY406050
CL11	Eggshell	*Psychrobacter* sp.	99	KU644214

CL12	Eggshell	*Psychrobacter* sp.	88	KY817998
CL20	Eggshell	*Psychrobacter* sp	100	KR029271
CL37	Eggshell	*Psychrobacter* sp.	98	KU501399
CL54	Eggshell	*Psychrobacter pulmonis*	99	KU364058
CL10	Eggshell	*Psychrobacter pulmonis*	99	KC866187
CL30	Eggshell	*Psychrobacter nivimaris*	100	KX027046
CL50	Eggshell	*Psychrobacter nivimaris*	100	KX027046
CL33	Eggshell	*Psychrobacter frigidicola*	96	KF712923
CL57	Eggshell	*Psychrobacter frigidicola*	99	KF712923
CL18	Eggshell	*Psychrobacter cibarius*	98	LK391538
CL22	Eggshell	*Psychrobacter cibarius*	99	LK391538
CL32	Eggshell	*Psychrobacter cryohalolentis*	99	CP022043
CL21	Eggshell	*Acinetobacter* sp.	100	JQ080653
CL15	Eggshell	*Acinetobacter* sp.	99	JQ080653
CL19	Eggshell	*Acinetobacter* sp.	99	JQ080653
CL14	Eggshell	*Acinetobacter* sp.	99	KP185134
CL26	Eggshell	*Acinetobacter* sp.	99	JQ080653
CL24	Eggshell	*Acinetobacter* sp.	100	AF336350
CL28	Eggshell	*Acinetobacter* sp.	99	JQ080653
CL29	Eggshell	*Acinetobacter* sp.	99	JQ080653
CL31	Eggshell	*Acinetobacter sp.*	99	AF336348
CL35	Eggshell	*Acinetobacter* sp.	99	JQ080653
CL36	Eggshell	*Acinetobacter* sp.	99	JQ080653
CL39	Eggshell	*Acinetobacter* sp.	99	JQ080653
CL44	Eggshell	*Acinetobacter* sp.	99	JQ080653
CL41	Eggshell	*Acinetobacter* sp.	99	JQ080653
CL91	Eggshell	*Acinetobacter baumannii*	99	JF919838
CL76	Eggshell	Uncultured *actinobacterium* clone	99	DQ829513
CL77	Eggshell	Uncultured *actinobacterium* clone	95	DQ829178
CL13	Eggshell	Uncultured bacterium clone	99	KU514944
CL47	Eggshell	Uncultured bacterium clone	99	KM456096
CL51	Eggshell	Uncultured bacterium clone	99	KU514973
CL61	Eggshell	Uncultured bacterium clone	98	AB657352
CL64	Eggshell	Uncultured bacterium clone	98	DQ800854
CL82	Eggshell	Uncultured bacterium clone	97	JQ387341
CL108	Egg content	Uncultured bacterial clone	91	JQ084406
CL1	Eggshell	*Staphylococcus equorum*	100	KX608723
CL3	Eggshell	*Staphylococcus haemolyticus*	99	KU977137
CL93	Eggshell	*Staphylococcus equorum*	99	KF439736
CL55	Eggshell	*Staphylococcus equorum*	99	CP013714
Cl89	Eggshell	*Staphylococcus equorum*	99	CP013114
CL62	Eggshell	*Staphylococcus epidermidis*	96	LT678237
CL52	Eggshell	Uncultured *Clostridiales* bacterium	99	AB702869
CL63	Eggshell	Uncultured *Clostridium* sp	96	KM244914
CL80	Eggshell	*Ralstonia* sp.	100	KU598712
CL67	Eggshell	Uncultured alpha *proteobacterium*	100	AF509578
CL68	Eggshell	Uncultured *Prevotellaceae* bacterium	97	FJ440089
CL59	Eggshell	*Lactobacillus helveticus*	100	LC062899
CL69	Eggshell	*Olsenella* sp.	99	LT635455

The clones CL13, CL47, CL51 matched sequences in Genbank with identity values of 99% to uncultured bacterium clone and showed a lower identity of 98% *Acinetobacter* sp. The clone CL61 showed a sequence identity of 98 % to uncultured bacterium clone, and showed a lower identity of 97 % to *Oscillibacter valericigenes*. *O. valericigenes* is Gram-negative, anaerobic was identified in alimentary canal of a Japanese corbicula clam (Iino *et al.*, 2007). The clone CL 64 showed a sequence identity of 98 % to uncultured bacterium clone, and showed a lower identity of 96 % to rhizosphere soil bacterium. The cloned isolate CL82 showed identity value of 97% to uncultured bacterium clone and lower identity of 96% to uncultured *Devosia* sp. In terms of the clone CL108 this showed identity of 91% to an uncultured bacterium clone and a lower identity of 90% to *Bacteroidales* bacterium.

3.7 Discussion and conclusion

Varied protocols of DNA isolation and purification from different types of organisms have been described (Li *et al.*, 2014; Rashdan, *et al.*, 2014). Generally, DNA extraction is a multi-step procedure comprising cell wall destruction, liberation of the cell contents and DNA purification. However, some of the methods used are time consuming and have high cost. In this study, a rapid cell lysis procedure (MBE) for nucleic acids isolation was compared with other procedures that involved using chemical treatments. The MBE method used was modified from the procedure performed by Reischl *et al*. (2000). The cell wall of Gram-positive bacteria, is more robust than the cell wall of Gram-negative bacteria, since it contains thick multilayered peptidoglycan in the outer cell wall which obstructs evacuating the cell content. Therefore, a method that is applicable to both types of bacteria is required, since the aim is to extract bacterial DNA from a mixed bacterial community. By comparing the five DNA extraction protocols on pure bacterial cultures, the MBE was found to be the most efficient method for producing PCR amplicons from a low number of bacterial cells (10^4) for both Gram-positive and Gram-negative bacteria. Other methods demanded more bacterial cells to obtain a PCR product. However, beside the advantage of MBE to be fast and simple, there are several limits regarding the size of the products which can be amplified. A study by Sepp *et al.* (1994) suggested that extraction of cell DNA by using water boiled method resulted in degrading the DNA and were found to be amplified up to 650 base pairs only with a better preservation of the target DNA.

It was noticed that the CE method was successful for extraction DNA from *E .coli* cells and a PCR band was clearly observed on the gel from a concentration of 10^6 cells/ml. However, no PCR amplicon was observed using the same method, and the same amount of bacterial cells with *S. equorum*. One reason may be that using Chelex-100 could form a thermal barrier that impedes weakening of the bacterial cell wall, since Gram-positive bacteria have thicker cell walls compared to Gram-negative bacteria, and extraction of the cell content including the genomic DNA will be more efficient in Gram-negative bacteria, which in turn may lead to better DNA amplification. On the other hand, the MBE was not an appropriate protocol for isolating DNA from egg content, since the method caused the egg proteins to solidify, making it difficult to purify DNA for use in PCR. Therefore, another method and procedure was developed that involved homogenisation and deproteinisation of the analysed sample to remove the excess protein. Using this method resulted in extraction of bacterial DNA from egg content and the generation of PCR products that can be used for cloning.

Previous studies have determined bacterial contamination of table eggs using conventional culture media and focused on studying bacteria that cause egg spoilage (Adesiyun *et al.*, 2005; De Reu *et al.*, 2009; Salihu *et al.*, 2015; Harry, 1963). However, none of the previous studies have attempted to study bacterial community in table eggs using the metagenomic approach. In this study we performed direct sequencing of the 16S rRNA gene from egg shell and egg content. Accordingly, 91 cloned isolates were identified from table eggs, by using the cloning technique for separating the extracted and amplified bacterial DNA fragments. The aim of this investigation was to study bacterial flora from table eggs using the molecular approach. Exploiting 16S rDNA cloning to describe the phylogenetic diversity in complex bacterial communities more comprehensively covers the population compared to cultivation. The phylogenetic data obtained in this study augments the diversity of bacteria from table eggs described previously (De Reu *et al*. 2005; Haines 1938; Alvarez-Fernández *et al*. 2012; De Reu *et al*. 2009; Jones *et al*. 2004).

Some bacterial species identified through the metagenomics approach were, however, not identified using the cultivation screening that was performed earlier in this study- *Psychrobacter* species, *Lactobacillus helveticus*, *Olsenella* sp., *Ralstonia* and

Prevotellaceae bacterium. The reason behind this might be attributed to the fact that some bacteria require specific growth requirements for cultivation and become visible on growth media. However, some interesting organisms have been identified such as *Psychrobacter* species which could be associated with the egg spoilage at low temperature. Gram negative bacteria are the most blamed organisms for egg spoilage (Haines, 1938). Both *Psychrobacter* and *Acinetobacter* which were found in high occurrence during this study are gram negative. Furthermore, and more importantly, *Acinetobacter* is considered as a life threatening microbe, since all species can resist a majority of known antibiotics (Peleg *et al*., 2008). *Staphylococcus*, *Clostridium and Lactobacillus* have been isolated previously from table eggs (Pyzik and Marek 2012; Stepień-Pyśniak, *et al*. 2009; Arathy *et al*. 2009). However, *Actinobacteria*, *Psychrobacter*, *Proteobacterium*, *Olsenella* and *Prevotellaceae* identified in this study have not been reported to be associated with table eggs.

It was found in the earlier part of this investigation using the culturing approach there is a high occurrence of *Staphylococcus* bacteria on the eggshells. On the other hand, by using a metagnomic approach *Psychrobacter* were found to be the dominant bacteria. One reason may be the DNA extraction method was not efficient since the Gram +ve bacteria have a thicker cell wall compared to Gram +ve bacteria, in which there may be a higher chance of gene amplification and detection of Gram –ve bacteria.

The study used the molecular method to produce a preliminary profile of the table egg microflora. As can be seen in Table 3-1, that a different distribution pattern for the major taxonomic grouping of bacteria in which the technique recovered 4 phylums of bacteria; Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes present in 92% of the cloned isolates, whereas the rest of the clones analysed were found to be uncultured clones. In overall, molecular approach has provided a dataset of table egg microbial flora that could not be identified using the culturing approach. However, identification of unknown bacteria using the culturing technique is difficult as suggested by (Achemedei, 2016). Therefore, using molecular approach for studying bacterial diversity in a food sample may provide information that could assist researchers in identifying unknown or uncultured bacteria. Nevertheless, Beside capability of the metagenomic approach to provide important information about uncultured bacteria, there are still some limitations that are worth noting. Each step in community analysis is open to bias or error, and the first place of the biases can occur during the extraction process in which some types of

bacteria may require particular chemical reagents or mechanical forces to evacuate their cell contents or to lyse the bacterial cell walls, thus, it may lead to a lower recovery rate (Farrelly *et al.*, 1995; Achemedei, 2016).

It can be concluded that MBE was found to be the best method for isolating bacterial DNA compared to the other procedures tested in this study. Isolating genomic DNA from a mixed bacterial community on eggshell was successfully performed using the MBE. A method for isolating bacterial DNA from a mixed bacterial community followed by a PCR and cloning approach is informative for identifying bacteria from mixed bacterial populations, and that, this technique can be applied to identifying bacteria from table eggs. In particular, *Psychrobacter* and *Acinetobacter* species were found in high occurrence in table eggs using the sequencing approach

CHAPTER 4 : Studying bacterial characteristics of *Psychrobacter* isolated from table eggs

4.1 Introduction

Several bacteria including *Psychrobacter* species have the ability to grow at low temperature (4°C) (Bakermans *et al.*, 2003).The genus *Psychrobacter* comprises psychrotolerant to psychrophilic, aerobic, Gram-negative, oxidase positive, non-motile, coccobacilli (Gini, 1990). Moreover, they are considered to be one of the most important lipolytic bacteria that contribute to nutritive and sensory changes of food (Bozal *et al.*, 2003). These spoilage organisms can be present in some types of food such as refrigerated meat and food during aerobic storage (Bozal *et al.*, 2003). *Psychrobacter* was found to be responsible for the short life of rehydrated salt-crude cod and production of rancid smell in all tested samples (Borch *et al.*, 1996). Also, they are opportunistic pathogens, and two clinical cases have been reported of ocular infection caused by *Psychrobacter immobilis* in a newborn who acquired the infection in the hospital (Gini, 1990), and another case in which *Psychrobacter arenosus* caused bacteraemia as a result of blood-transfusion (Caspar *et al.*, 2013).

Growth of *Psychrobacter* spp has been achieved on a tryptone soya agar (TSA) medium at 35 °C (Bozal *et al.*, 2003). However, they are capable of growing at temperatures from -10 to 37 °C. Many species can grow optimally at 25-30 °C. These microbes apparently have the ability to survive and grow at low temperatures, which makes it critical to consider the possible adverse effects from a food quality perspective. In 1986, the genus *Psychrobacter* was first introduced to describe a group of mainly psychrophilic bacteria that are commonly isolated from fish, processed meat and poultry (Juni and Heym, 1986). Subsequently, *Psychrobacter* strains have been isolated from orinthogenic soils, anchor grease, ice, and ice algae biomass. Moreover, they have been isolated from gills and intestines of fish (Bowman, Nichols and McMeekin, 1997; Maruyama *et al.*, 2000). *Psychrobacter faecalis* was isolated from bio-aerosols originating from pigeon faeces (Kämpfer *et al.*, 2002). *Psychrobacter maritimus* was initially isolated from coastal sea-ice and sediment samples (Romanenko *et al.*, 2004). Types of *Psychrobacter* that have been isolated from other habitats are described in Table 4.1.

In this study, *Psychrobacter* was found to be the most commonly identified more gene by analysis of 16s rRNA sequences from both eggshell and egg content. Also, *Psychrobacter* species identified in this study have not been reported from table eggs in previous studies,

and also were not isolated using conventional culture technique in this study. It is therefore of interest to attempt to isolate these bacteria from table eggs using culture media and the appropriate growth requirements.

4.2 Objective

The objective of this study was to isolate the *Psychrobacter* from table eggs using culturing techniques. *Psychrobacter* can grow at low temperatures, and is considered as a critical contaminant that may spoil eggs even at fridge temperatures. Therefore, isolated *Psychrobacter* strains have been tested for biochemical and growth characteristics, including temperature and salinity tolerance.

Table 4-1 Bacterial characteristics, source of isolation and diseases caused by *Psychrobacter* species.

Psychrobacter isolates	Source of isolation	Growth temp max	Tolerance to 12% NaCl	Disease causes	Year of isolation	Reference
Psychrobacter immobilis	Fish, meat and poultry	35 °C	+	Ocular infection in human	1990	Gini (1990)
Psychrobacter faecalis	Pigeon faeces	36 °C	+	ND[a]	2002	Kämpfer *et al*. (2002)
Psychrobacter maritimus	costal-sea ice sediment	37 °C	-	ND	2004	Romanenko *et al*. (2004)
Psychrobacter pulmonis	lamb lung	37 °C	-	Lung infection in sheep	2003	Vela *et al*. (2003)
Psychrobacter jeotagli	Fermented food, jeotgal	37 °C	-	ND	2005	Jung (2005)
Psychrobacter galcincola	Sea ice cores	22 °C	+	ND	1997	Bowman *et al*. (1997)
Psychrobacter arenosus	costal sea ice, human blood	37 °C	-	Bacteraemia in human	2006	Leung *et al*. (2006); Romanenko *et al*. (2004)
Psychrobacter arcticus	Siberian permafrost	22 °C				Bakermans *et al*. (2006)
Psychrobacter cibarius	Fermented food, jeotgal	37 °C	-	ND	2005	Jung (2005)

[a]ND: non-determined

4.3 Materials and methods

4.3.1 Isolation of *Psychrobacter* strains

Bacterial colonies were isolated from eggshell as described in section 2.3.3. and were plated on tryptone soya agar (TSA). For isolating bacteria from the egg content, the previous methodology explained in section 2.3.4 was followed. All the plates were incubated at 4 °C for 4 days.

4.3.2 DNA extraction and PCR amplification

For identifying the isolated bacteria using PCR, genomic DNA was extracted using the boiling method. One single colony was inoculated into 20 µl sterilized ddH_2O, and mixed by vortex. The suspension was incubated at 100 °C for 10 minutes. Thereafter, the sample was placed on ice for 3 minutes, and centrifuged at high-speed 13,000 x for 10 minutes. The cell lysates were placed at -20 °C until further treatment.

The 16S rDNA primers used in this study were 9F (5′-GAGTTTGATCCTGGCTCAG-3′; position 9-27, *Escherichia coli* 16S rRNA numbering) and 536R (5′-GTATTACCGCGGCTGCTG-3′; position 536-519). These were previously utilised by Kim *et al*. (2004) for detection of bacterial isolate from water samples. PCR reaction was performed in a 50 µl PCR tube, with 25 µl 2x BioMix buffer (Bioline), 1µl forward primer (10 µM 9F RNA), 1 µl reverse primer (10 µM 536R RNA) and 2 µl cell lysate. The PCR mixture was heated to 95 °C in a thermal cycler for 4 min, the PCR program was set on 30 reaction cycles of 95 °C for 30 sec, followed by the annealing step at 54°C for 30 sec, then the elongation step at 72 °C for 30 sec, and finally one extension cycle at 72 °C for 7 min.

4.3.3 Detection of the PCR products

The PCR products were run on a 1% (w/v) agarose gel to detect the DNA amplification product. The gel was prepared as described previously in section 2.3.9.

4.3.4 Analysis of 16S rRNA gene sequence

Samples were treated as described in section 2.3.1.1.

4.3.5 Phylogenetic analysis

In order to construct a phylogenetic tree, the obtained sequence results were aligned using a multiple alignment tool, then the tree was constructed by applying a neighbour-joining method. Sequences for comparison were retrieved from the NCBI database, pre-aligned with the CLUSTALW tool. The phylogenetic tree was constructed using MEGA 6 software (Tamura *et al.*, 2013).

4.3.6 Temperature tolerance test

Bacterial isolates were streaked on TSA, and incubated at different temperatures, ranging from 10 to 40 °C for 48 h and at 4 °C for 4 days. In order to determine the maximum growth temperature of *Psychrobacter faecalis* and *Psychrobacter martimus*, 10 µl of overnight culture [$\sim 10^9$ cells. ml^{-1}] was inoculated into 190 µl TSB in microtiter plates and incubated at temperatures between 30 and 40 °C for 24 h. Growth at 0 and 24 h was measured as OD_{620} using a SpectraMax® M5 microplate reader.

4.3.7 Salinity tolerance test

For determining the salinity tolerance, 190 µl of tryptone soya broth medium containing different NaCl concentrations from 0 to 20% (W/V) were inoculated with 10 µl of overnight *Psychrobacter* culture [$\sim 10^9$ cells ml^{-1}] and mixed in a microtiter plate, and incubated at 25 °C for 24 h. Blank reading was performed at 0 h for the samples directly after inoculation. Growth was monitored using the SpectraMax® M5 after 24 h.

4.3.8 Oxidase and catalase production

For oxidase production test, a single colony from an overnight culture on TSA was streaked on a piece of Whatman filter paper (No.1), moistened previously with 1% solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma-Aldrich). A positive reaction for oxidase was determined visually, in which a purple colour was obtained within 30 seconds. In terms of catalase production test, a single colony was smeared on a glass slide containing a drop of 5 % H_2O_2 solution. Rapid evolution of bubbles due to molecular oxygen being released was an indication of a positive catalase production.

4.3.9 API 20NE rapid identification systems

In order to study the characteristics of the isolated bacteria, they were tested using the substrates utilization strips of API 20NE for the identification of non-enteric Gram-negative rods following the supplier's instructions (Bio-M Bio-Mérieux, Basingstoke, Great Britain). Therefore, a single colony was inoculated in 5 ml sterilised water to prepare the bacterial inoculum. The API strip was filled with inoculum and incubated at room temperature. The reading was obtained at 24 and 48 h. Results were recorded as positive or negative, scored against the reading table in the instruction sheet (= interpretive colour chart).

4.4 Results

4.4.1 TVCs of psychrophiles isolated from table eggs

After *Psychrobacter* was found according to direct analysis of the 16S rRNA gene that was performed earlier in this study, it was then of interest to isolate these bacteria by providing the appropriate growth medium and conditions. Therefore, bacteria were isolated from eggshells and egg content on TSA as described in the methodology, after incubation at 4 °C for 4 days. Incubating samples at 4 °C will allow psychrophiles to grow and suppress other organisms from growing on plated media. The results showed that bacteria isolated from eggshell were observed (Figure 4.1). TVCs was estimated to be 4.5 x 10^3 CFU/eggshell. On the other hand, no growth was observed for the egg content samples.

A B

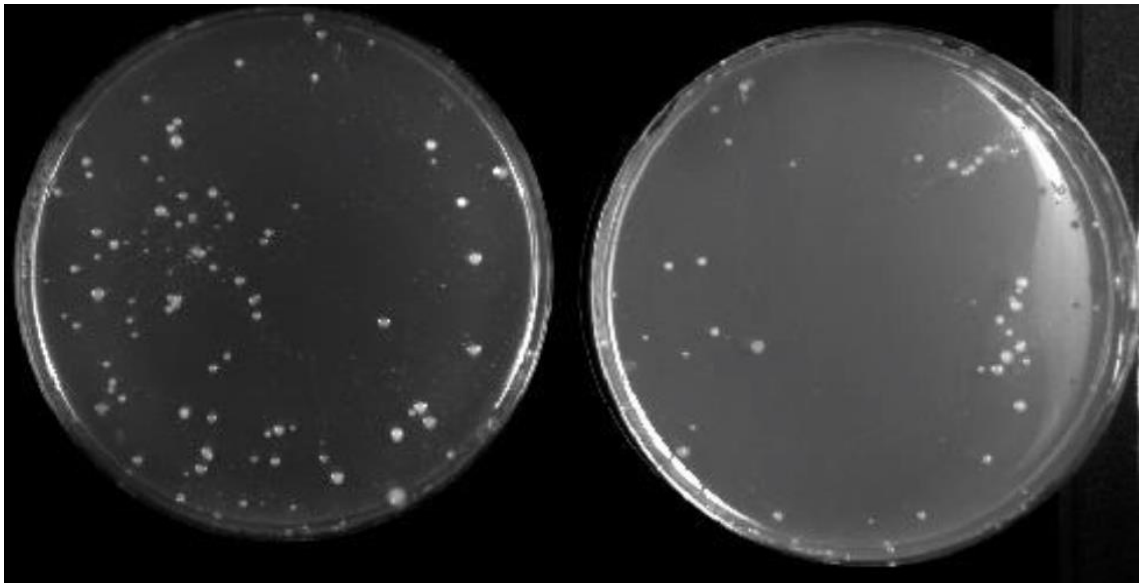

Figure 4.1 Bacterial growth isolated from egg shell and incubated at 4 °C for 4 days. The figure shows bacterial growth from two different eggshells. The mean value of TVCs was estimated to be 4.5 x 10^3 CFU/egg shell (*n*=3, S.D=1734).

The colony morphology for the majority of bacterial isolates were circular in shape, with smooth texture and creamy colour. Other isolates were found to be irregular in shape, with rough textures and moderate in size. A total number of 11 isolates were randomly

selected based on morphological variations that represented the observed bacterial colonies.

4.4.2 Identification of bacterial egg isolates based on 16S rDNA sequence analysis

In order to identify the bacterial isolates by determining the 16S rRNA sequence, a PCR was performed and the amplicons were run on a gel. As can be seen from Figure 4.2, amplified 16S rDNA amplicons of the bacterial strains were located at the right size, about 590 bp.

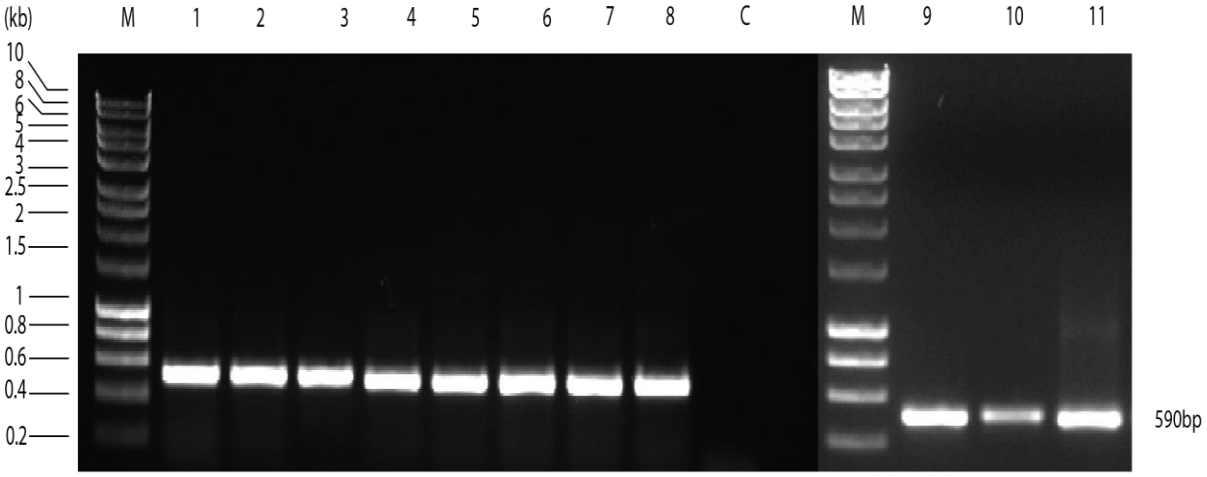

Figure 4.2 Agarose gel showing amplified part of 16S rRNA gene from single bacterial colonies isolated from eggshell by incubation on TSA 4 °C. Lane M: hyperladder I (Bioline), lane 1:11 DNA amplification of bacterial strains. Lane C is a negative control that was performed without template DNA.

The PCR products were sequenced using Sanger sequencing, and the results were analysed using the NCBI tool BLASTN. Among the 11 isolates, the results revealed that two different bacterial species were identified *Psychrobacter maritimus* and *Psychrobacter faecalis.* The sequence of the strains P1 and P9 shared between 99-100% identity to the gene of *Psychrobacter maritimus*. The sequence of the strains P2, P3, P4, P5, P6, P7, P8, P10 and P11 shared an identity of 99 – 100% to the gene of *Psychrobacter faecalis* (Table 4.2). In terms of *Psychrobacter faecalis* 8 isolates (P2, P3, P4, P5, P6, P7, P8 and P10) were found identical matching the gene sequence from Genbank of (KX650120).

Table 4-2 Colony morphology and identification of *Psychrobacter* isolates

Strain	Culture characteristics						Bacterial Strain	Identity %	Accession No.
	Shape	Margin	Elevation	Surface	Colour	size			
P1	Irregular	Undulate	Raised	Rough	Cream	Moderate	*Psychrobacter maritimus*	99%	KJ939482
P2	Circular	Entire	Raised	Smooth	Cream	Moderate	*Psychrobacter faecalis*	99%	KX650120
P3	Circular	Entire	Raised	Smooth	Cream	Moderate	*Psychrobacter faecalis*	99%	KX650120
P4	Circular	Entire	Raised	Smooth	Cream	Moderate	*Psychrobacter faecalis*	99%	KX650120
P5	Circular	Entire	Raised	Smooth	Cream	Moderate	*Psychrobacter faecalis*	99%	KX650120
P6	Circular	Entire	Raised	Smooth	Cream	Moderate	*Psychrobacter faecalis*	100%	KX650120
P7	Circular	Entire	Raised	Smooth	Cream	Moderate	*Psychrobacter faecalis*	99%	KX650120
P8	Circular	Entire	Raised	Smooth	Cream	Moderate	*Psychrobacter faecalis*	99%	KX650120
P9	Irregular	Undulate	Raised	Rough	Cream	Moderate	*Psychrobacter maritimus*	100%	HM584045
P10	Circular	Entire	Raised	Smooth	Cream	Moderate	*Psychrobacter faecalis*	99%	KX650120
P11	Circular	Entire	Raised	Smooth	Cream	Moderate	*Psychrobacter faecalis*	99%	KT767856

As can be seen from the table, the majority of the isolates shared an identity of ≥ 99% to the species *Psychrobacter faecalis*. The species *Psychrobacter maritimus* can be discriminated by their distinct colony morphologies that is irregular in shape and rough texture.

4.4.3 Growth characteristics and biochemical tests

The bacterial isolates were subjected to various growth and biochemical tests, to identify the optimal, maximum and minimal growth temperatures, and to determine salinity tolerance. These characteristics were compared to the phenotypic characteristics of *P. maritimus* and *P. faecalis* strains isolated in previous studies (Gini, 1990; Kämpfer *et al.*, 2002; Romanenko *et al.*, 2004; Jung, 2005; Bakermans *et al.*, 2006; Deschaght *et al.*, 2012). *P. faecalis* was found to be able to grow at maximum temperature of 36 °C and up to 12% NaCl (Kämpfer *et al.*, 2002), whereas *P. maritimus* was found to be able to grow at maximum temperature of 37 °C and with no growth at 12% NaCl.

4.4.4 Description of *Psychrobacter maritimus* isolates.

P.maritimus strains isolated in this study are aerobic, Gram negative, coccobacilli. The colonies have an irregular shape, undulate margin and creamy colour (Figure 4.3). They are oxidase and catalase positive, and psychrotolerant. On TSA good growth was observed between 4-38° C. The strain did not grow at 39-40 °C (Figure 4.4A). Sodium ions are not required for growth; the strains tolerate 0-10% (w/v) NaCl, but are not able to grow in 15% NaCl (Table 4.4). Acid was not formed from carbohydrate. Metabolic reactions are described in Table 5.5. The strains are positive for nitrate reduction, but negative for urease, L-leucine, arginine, aesculin, indole production, ß-galactosidase and gelatinase, according to the API substrate panel reactions. Both of the strains were positive for assimilation of L-arabinose, adipic acid and malate, but were negative for D-glucose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, caprate, citrate, and phenyl acetate (Table 4.5). However; a study by Romanenko *et al.* (2004) the maximum growth temperature for *P. maritimus* was found at 37 °C and the strain was able to grow at 12% NaCl. The utilisation of L-Arabinose was found negative whereas in our study positive.

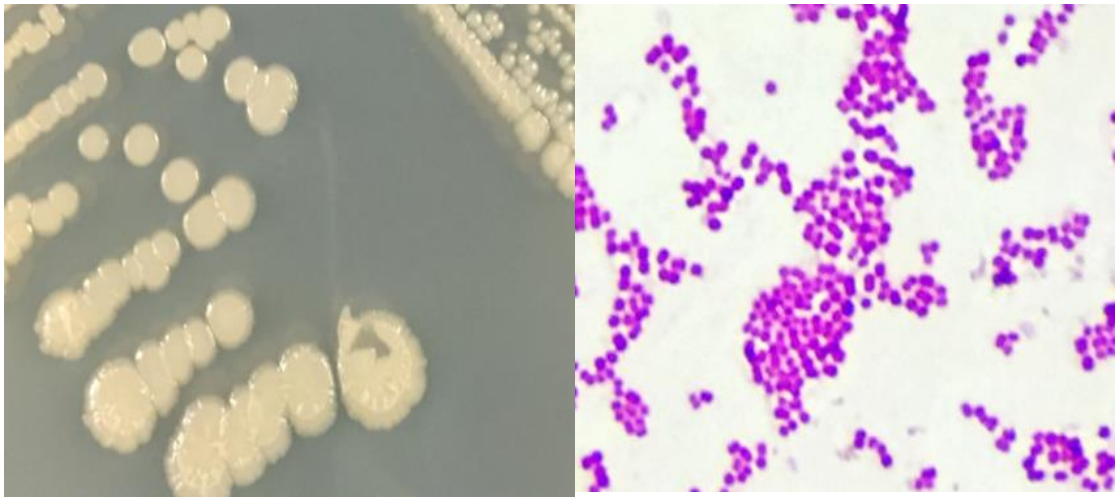

Figure 4.3 Bacterial colony and cell morphology of *Psychrobacter maritimus* (P1), the magnification was at 100x.

Table 4-3 Growth temperatures and salinity tolerance tests of the isolated bacterial strains

Character	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**	**P11**
Growth temperature											
4 °C	+	+	+	+	+	+	+	+	+	+	+
10 °C	+	+	+	+	+	+	+	+	+	+	+
15 °C	+	+	+	+	+	+	+	+	+	+	+
20 °C	+	+	+	+	+	+	+	+	+	+	+
30 °C	+	+	+	+	+	+	+	+	+	+	+
40 °C	-	-	-	-	-	-	-	-	-	-	-
Salinity tolerance											
0%(W/V) NaCl	+	+	+	+	+	+	+	+	+	+	+
2% (W/V) NaCl	+	+	+	+	+	+	+	+	+	+	+
4% (W/V) NaCl	+	+	+	+	+	+	+	+	+	+	+
6% (W/V) NaCl	+	+	+	+	+	+	+	+	+	+	+
8% (W/V) NaCl	+	+	+	+	+	+	+	+	+	+	+
10% (W/V) NaCl	+	+	+	+	+	+	+	+	+	+	+
15 % (W/V) NaCl	-	+	+	+	+	+	+	+	-	+	+
20% (W/V) NaCl	-	-	-	-	-	-	-	-	-	-	-

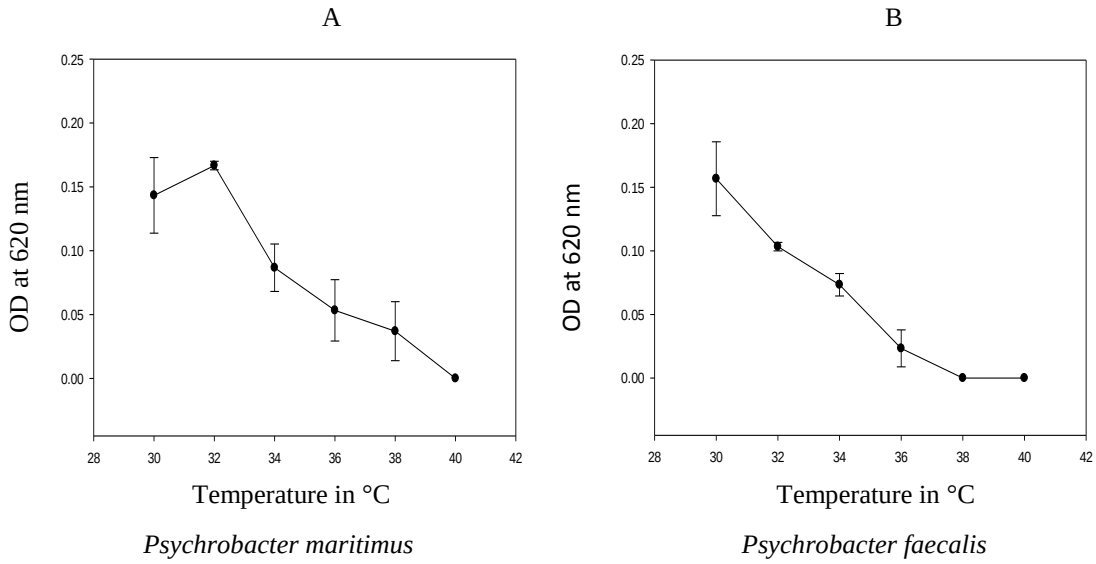

Figure 4.4 Growth of *Psychrobacter maritimus* and *Psychrobacter faecalis* after incubating at different temperatures for 24 hours. The strains tested were P1 and P6.

4.4.5 Description of *Psychrobacter faecalis*

P. faecalis strains are aerobic, Gram negative, coccobacilli, that have a regular shape colony with entire edge and smooth surface. The colony colour is creamy and moderate in size (Figure 4.5). The bacteria are oxidase- and catalase positive. They are psychrotolerant, growing at 4-37 °C. In addition, the strain does not grow at 39-40 °C (Figure 4.4B). Sodium ions are not required for growth, the strains were able to grow in a medium containing 0-15% (w/v) NaCl, but not in 20% NaCl (Table 4.4). Acid was not formed from carbohydrate. The strains were positive for nitrate reduction, but was negative for urease, arginine, gelatinase, indole production and ß-galactosidase and gelatiase according to the result of the API substrate panel reactions. The strains were positive for assimilation of malate, and weakly positive for adipic acid and L-arabinose, but was negative for assimilation of D-glucose, D-mannitol, D-mannose, N-acetyl-glucosamine, D-maltose, potassium glucose, caprate, citrate and phenyl acetate (Table 4.5).

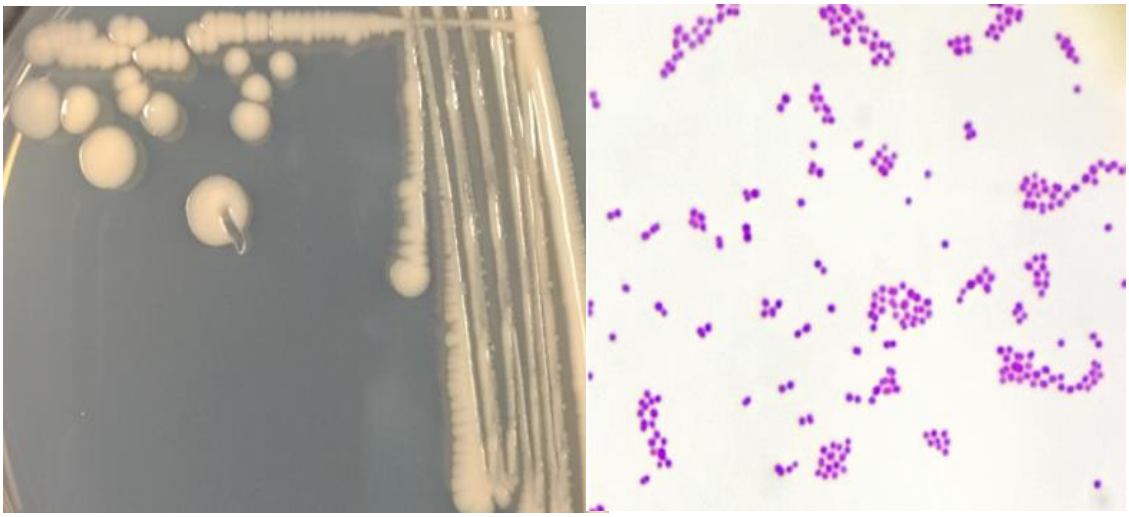

Figure 4.5 Bacterial colony and cell morphology of *Psychrobacter faecalis* (P6), the magnification was at 100x.

Table 4-4 Phenotypic characteristics of *Psychrobacter* strains isolated from table eggs, and additional *Psychrobacter* species reported in literature.

Characteristic	* *P. maritimus*	** *P. faecalis*	[a] *P. maritimus*	[b] *P. faecalis*	[c] *P. proteolyticus*	[d] *P. immobilis*	[e] *P. glacinocola*	[f] *P. pacificensis*	[g] *P. uratiovorans*	[h] *P. phenylpyruvicus*	[i] *P. arenosus*
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	ND	-	+	V+	-	V-	+	-
Arginine dihydrolase	-	-	-	-	-	-	V-	-	+	+	-
Urease activity	-	-	V+	-	+	(+)	-	-	V+	+	-
Esculin hydrolysis	-	-	-	-	-	-	-	-	-	-	-
Acid from carbohydrate	-	-	-	-	-	+	-	+	-	-	
Gelatinase	-	-	-	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	-	-
ß-galactosidase	-	-	-	-	-	-	-	-	-	-	-
Growth temp. (max.)	38°C	37°C	37°C	36°C	35°C	25°C	22 °C	33°C	25-27°C	39°C	37 °C
Growth temp. (optimal)	25-32°C	25-30°C	25-28°C	15-30°C	ND	20 °C	13-15°C	25°C	17-19°C	32°C	22 °C
Growth at 37 °C	+	+	+	+	-	-	-	-	-	-	+
Salinity tolerance											
8% (W/V) NaCl	+	+	+	+	+	+	+	+	+	+	+
10% (W/V) NaCl	+	+	+	+	+	+	+	+	+	+	+
15% (W/V) NaCl	-	+	-	+	+	-	+	-	-	-	+
Carbon sources utilised											
D-glucose	-	-	-	(+)	-	+	-	-	-	-	-
L-Arabinose	+	(+)	-	(+)	-	ND	-	-	-	-	V-
D-mannose	-	-	-	ND	ND	+	-	-	ND	-	-
D-mannitol	-	-	-	ND	-	ND	ND	-	ND	-	-
N-acetyl-glucosamine	-	-	-	+	ND	ND	-	-	ND	-	-
D-maltose	-	-	-	+	ND	ND	ND	-	ND	ND	-
Potassium gluconate	-	-	-	ND	ND	ND	ND	-	ND	-	-
Caprate	-	-	-	ND	-	ND	ND	-	ND	ND	-
Adipic acid	+	(+)	V+	ND	-	ND	ND	-	ND	ND	-
Malate	+	+	V-	+	-	ND	-	+	-	+	+
Citrate	-	-	-	+	+	-	V+	-	-	+	-
Phenyl acetate	-	-	-	-	-	ND	-	-	-	-	-

[a]Data from Romanenko *et al*. (2004); [b]Kämpfer *et al*. (2002); [c]Denner *et al*. (2001); [d]Juni and Heym (1986); [e]Bowman *et al*. (1997); [f]Maruyama *et al*. (2000); [g]Bowman *et al*. (1996); [h]Deschaght *et al*. (2012); [i]Caspar *et al*. (2013). * P1 and P9; ** P3, P4, P5, P6, P7, P8, P10 & P11 +, positive; (+), weakly positive; -, negative; ND, no data; V+, 11-89% are positive and the type strain is positive; V-, 11-89% are positive and the type strain is negative.

4.5 Discussion

The majority of *Moraxella*-like psychrophiles isolated from the environment are phylogenetically positioned close to the *Psychrobacter* clade in the family *Moraxellaceae*, which comprises three genera -*Moraxella*, *Acinetobacter* and *Psychrobacter* (Maruyama *et al.*, 2000). The phenotypic features of psychrophilic strains of *Psychrobacter maritimus* isolated in this study almost coincided with results obtained by Romanenko *et al*. (2004); that is, the strain KMM3646^T is Gram-negative, aerobic, oxidase and catalase-positive. In addition, the strains were found to be negative for hydrolysing arginine, and esculin, indole production, gelatinase, β-galactosidase, and carbon sources not utilised were D-glucose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, gluconate, citrate, and phenyl acetate. The isolates were positive for nitrate reduction, tolerating 10% (W/V) NaCl. However, urease activities were found variable. Other *Psychrobacter* species, *P. faecalis*, *P. glacinocola*, *P. pacificensis* and *P. arenosus* do not utilise urea (Table 4.5). Romanenko *et al*. (2004) reported that the maxmium temperature for *Psychrobacter maritimus* to grow is 37 °C, however, in this study *P. maritimus* P1 was able to grow at 38 °C (Figure 4.4).

With regards to the strains of *Psychrobacter faecalis*, the phenotypic characteristics were found to be similar to the strain M4 10^T described by Kämpfer *et al*. (2002), in terms of oxidase, catalase, arginine dihydrolase, urease, esculin, acid from carbohydrate, gelatinase, indole production, β-galactosidase, tolerating 15% NaCl, utilising L-arabinose, malate and phenyl acetate. On the other hand, differences were noticed, in the ability of M4 10^T strain to utilise D-glucose, D-maltose, citrate, and N-acetyl-glucosamine.

All strains grew well at room temperature (25 °C), and could survive and grow at 4 °C, which might adversely affect the egg quality, since they able to grow at low temperature. However, in the UK most table eggs are stored at room temperature, which is considered an optimal temperature for *Psychrobacter* to grow. In other countries particularly the ones that have warm weather, eggs tend to be stored in the fridge at around 4 °C. At this temperature *Psychrobacter* can still grow, but growth is very slow, and therefore, longer egg shelf life might be obtained.

In conclusion, the isolated *Psychrobacter* strains should be considered critical bacteria that are able to grow at low temperature and in the presence of > 10 % NaCl. In addition, All isolated strains were found to be resistant to erythromycin, and trimethoprim. On the other hand, all the isolates were sensitive to ampicillin and gentamycin.

CHAPTER 5 : Concluding discussion

Bacterial spoilage of the egg occurs generally as result of the penetration of the shell by bacteria that are deposited on the surface of the egg particularly after it has been laid on contaminated surfaces (Harry, 1963). The undesirable changes in eggs differ depending on the ability of bacteria to penetrate the shell and being able to overcome the antibacterial properties of the egg (Lutsky and Bell, 1953). It has been demonstrated that the cause of egg spoilage occurs commonly from the growth of Gram negative bacteria within the egg content (Board and Tranter, 1995). Also, *Psychrobacter* was found to be responsible for the short life of rehydrated salt-cured cod and production of rancid smell in all tested samples (Borch *et al*., 1996). Bacterial outbreaks and food poisoning issues occurred in the last decade due to consumption of undercooked food (Rocourt *et al*., 2003).

Prior work has documented the importance of studying bacterial diversity of table eggs, and how the presence of these organisms may affect the quality of eggs, and pose a threat to public health (De Reu *et al*., 2009; Alvarez-Fernández *et al*., 2012). However, these studies have been performed using traditional culture-based techniques rather than molecular biological approaches. In particular they have not focused on the sequencing approach for identifying uncultured bacteria. In this project both cultural and sequencing approaches were used for studying bacterial diversity of table eggs, in order to identify a wider range of the organisms present.

It has been reported that the extent of eggshell contamination ranges from 10^2 up to 10^7 CFU/eggshells (Board and Tranter, 1995). In this study it was found that TVCs of bacteria from eggshell were 2 x 10^5 to 5.7 x 10^5 CFU/eggshell from different housing systems, similar to values reported by De Reu *et al*. (2008) and Lucore *et al*. (1997), but lower than the TVCs reported by Alvarez-Fernández *et al*. (2012). However, many factors can affect the recovery of bacteria from eggshell, including egg washing, storage condition and housing systems. Washing of class A eggs to remove dirt and faecal material is not allowed in the UK. Nevertheless, some studies were performed before the washing ban in 1995 (Haines 1938; Board and Tranter 1995). Also, the treatment technique of isolating bacteria from eggshell may influence the result of microbial counts, for example some studies used the swab technique and the TVCs were calculated per cm^2 (Alvarez-Fernández *et al*., 2012). Therefore, TVCs of bacteria from eggshell would vary depending on these variables.

Lower TVCs of bacteria were observed in egg content of caged system 4.2 x 10^2 CFU/ml compared to organic free range eggs. The lower numbers may be attributed to the fact that eggshell is more prone to environmental contamination in free range system than in caged system. Moreover, food animals produced organically have the ability to contain higher rate of bacterial contamination than those produced conventionally, since the usage of antibiotic in organic systems is prohibited (Winter and Davis, 2006)

There was no significant difference found between TVCs of bacteria isolated from different housing systems for either eggshell or egg content. However, free range eggs tended to have higher microbial load on the shell and in the content than the caged system eggs. One reason may be that hens in the free range systems lay their eggs in the open environment, which will contain more contaminants such as faeces and dirt. Thus, free range eggs are more prone to become contaminated than caged eggs laid on clean surfaces.

In terms of isolation by culturing, *Staphylococcus* and *Micrococcus* were found to be the major contaminants of both eggshell and egg content. Other bacterial flora were also isolated from eggs including species of *Bacillus*, *Brevundimonas, Stenotrophomonas*, *Kocuria*, *Acinetobacter*, *Corynebacterium*, *Brachybacterium*, *Morexella, Brevibacterium* and *Chryseobacterium*. Some of these bacterial genera have been reported previously to be found in eggs (Arathy *et al*. 2009; Chaemsanit *et al*. 2015)

Most importantly *Salmonella enteritidis*, considered as a critical pathogen that causes food poisoning and illness in humans as a result of consuming contaminated eggs, was not isolated among any of the samples tested. This could be as a result of the monitoring control systems that have been applied by both farmers and food safety organisations to control the incidence of *Salmonella* in eggs, including the vaccination programs that are applied in the UK to the hens to control the spread of *Salmonella*. However, other pathogens such as *Clostridium perfringens* were found at a level of 9 x 10^2 CFU/eggshell. This level of contamination may cause illnesses. *C. perfringens* is considered as one of the most common causes of foodborne illness in the US, being estimated to cause nearly 1 million illnesses each year. In addition, the number of food outbreaks reported in 2013 was 16 in the UK. Thus, it is essential that the hazard of this bacterium should be assessed

and action to prevent food outbreaks as a result of consuming contaminated eggs should be taken.

There could be a vast majority of as yet unidentified bacteria, some of which may still be viable and cause illness if consumed by humans, but these bacteria may be non-culturable, or may require particular growth requirements in order to grow in laboratory media. A cloning approach is one way to access the 16S rDNA genes without culturing the organisms (Akkermans *et al*., 2001). The advantage of using cloning is the ability to separate DNA fragments following amplification of an environmental sample. Nevertheless, it is true that extracting bacterial DNA directly from food samples can be challenging. This is the first study to my knowledge, of bacterial diversity of table eggs using a sequencing approach in order to identify VBNC organisms. The results provided evidence for the presence of bacteria from table eggs, which had not been identified using a conventional culturing approach.

Before cloning individual fragments, DNA from eggshell and egg content had to be extracted and amplified using a high fidelity enzyme. Some published papers have described extracting DNA directly from soil, water and other environmental samples including food (Leff *et al.*, 1995a; Krsek and Wellington, 1999; Mudariki *et al.*, 2013; Omar, Atif and Mogahid, 2014), but no studies have focused on extracting bacterial DNA from eggshell or egg content. Extraction of bacterial DNA from egg shell was achieved by applying a modified boiling extraction method (MBE). The modifications were in reducing the incubation temperature from 100 °C to 85 °C and increasing the incubation time from 10 minutes to 20 minutes. However, reducing the extraction temperature and increasing the incubation time was found to slightly but not significantly improve the DNA yield obtained.

Comparing with four other cell lysate methods, the MBE was found to be the fastest and most efficient in terms of obtaining higher DNA yields after the amplification step. On the other hand, the MBE was not the appropriate choice for extracting DNA directly from egg content, since it relies on high temperature for lysing bacterial cells. Using high temperature in extracting bacterial DNA from egg content samples results in solidifying egg protein. Consequently, it becomes difficult to separate bacterial DNA from the organic materials. Therefore, another method was developed which relied on chemical

and mechanical treatments for lysing the bacterial cell wall and purifying bacterial DNA from the bulk of organic substances. Potentially, this method can be applied for extracting bacterial DNA directly from any food sample that contains high levels of organic materials such as protein.

After bacterial DNA had been successfully extracted, and PCR amplified using the Strata Clone enzyme, amplified products were then cloned in order to separate the individual DNA molecules. The concept of this approach was to ligate bacterial DNA fragments into linearised plasmids. Subsequently, competent host bacteria were transformed with the plasmids (Akkermans *et al*., 2001). However, some limitations are worth noting, such as the time required for selecting positive transformants and confirming the presence of gene inserts. Another limitation lies in the ability to randomly select transformants from a set of identical colonies, in order to ensure that the same amplified gene is not isolated and sequenced repeatedly, which of course is not efficient in terms of cost and time.

The results obtained by using the cloning approach suggested the presence of *Psychrobacter* species in high abundance both on eggshell and in egg content. *Psychrobacter* species were not isolated by the earlier culturing approach, and the reason is that they required particular growth conditions for them to grow optimally. *Psychrobacter* are Gram-negative, aerobic, psychrotolerant, non-pigmented and non-motile bacteria (Romanenko *et al.*, 2004). It has been shown that *Psychrobacter* grow efficiently on TSA medium and can grow at low temperatures (Vela *et al.*, 2003; Romanenko *et al.*, 2004; Jung, 2005). It was claimed in a previous study that Gram-negative bacteria are the most common organisms that spoil and cause rotten eggs (Haines, 1938). In this study *Psychrobacter* was found in high occurrence from both eggshell and egg content, and it might be one of the critical organisms that cause egg spoilage.

It should also be noted of the limitations and biases inherent to a metagenomics approach. Biases can occur in many stages including at the first place of DNA recovery, in which the procedure used may not recover total DNA and this attributed to the efficiency of each cell lysis techniques. The second place a bias may occur at the amplification step and this can be minimised by using high fidelity enzyme and reducing the number of amplification cycles.

From a food safety perspective these organisms could be considered important, since they can grow at a low temperature of 4 °C, which is the fridge temperature that is expected to preserve food. Pathogenicity of *Psychrobacter* species is still poorly understood, and the species *Psychrobacter immobilis* that was described by Juni and Heym (1986) has been found to be pathogenic and to cause ocular infection in infants and bacteraemia in adult humans (Gini, 1990; Caspar *et al.*, 2013). The *Psychrobacter faecalis* and *Psychrobacter maritimus* strains isolated in this study were found to be slightly different in their phenotypic characteristics compared to the previous studies (Romanenko *et al*., 2004; Kämpfer *et al*., 2002), but generally conformed to the earlier known characteristics.

This study has therefore indicated the benefits of using molecular techniques for identifying bacteria from table eggs. Future work may involve conducting more experiments to provide more information about pathogenicity of *Psychrobacter* bacteria associated with eggs, and whether they are a cause of egg spoilage. Also, the remaining 109 cloned samples that were prepared and kept in -20 can be sequenced and analysed which might reveal other interesting bacteria that are worth investigation.

Recommendations for further research

1- In depth exploration of the influence of housing system on total microbial load from both eggshells and egg content.
2- Increase the number of egg samples by considering each batch of eggs to be one spooled sample.
3- It would also be helpful to identify another DNA extraction technique for recovering bacterial DNA from a low number of bacterial community.
4- Consider using a full-length of 16S rRNA rather than a partial gene particularly in the cloning approach.

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APPENDICES

Appendix I TVCs from both eggshell and egg content that were plated on PCA and analysed in the study

Egg No.	Date	Grade	Best before date	Agar name	Housing system	[a]Egg source	Incubation temp	TVCs eggshell	TVCs egg content
1	06/08/2012	A	21/08/2012	PCA	CE	A	30 °C	1367000	3100
2	06/08/2012	A	21/08/2012	PCA	CE	A	30 °C	3020000	1900
3	06/08/2012	A	21/08/2012	PCA	CE	A	30 °C	302000	2100
4	13/08/2012	A	29/08/2012	PCA	OFRE	B	30 °C	40000	200
5	13/08/2012	A	29/08/2012	PCA	OFRE	B	30 °C	3420000	0
6	13/08/2012	A	29/08/2012	PCA	OFRE	B	30 °C	230000	0
7	20/08/2012	A	04/09/2012	PCA	FRE	C	30 °C	10000	700
8	20/08/2012	A	04/09/2012	PCA	FRE	C	30 °C	115000	0
9	20/08/2012	A	07/09/2012	PCA	FRE	C	30 °C	385000	0
10	28/08/2012	A	07/09/2012	PCA	CE	D	30 °C	80333	0
11	28/08/2012	A	07/09/2012	PCA	CE	D	30 °C	7000	0
12	28/08/2012	A	07/09/2012	PCA	CE	D	30 °C	0	0
13	05/09/2012	A	15/09/2012	PCA	FRE	E	30 °C	101500	0
14	05/09/2012	A	15/09/2012	PCA	FRE	E	30 °C	435000	0
15	05/09/2012	A	15/09/2012	PCA	FRE	E	30 °C	196500	0
16	11/09/2012	A	24/09/2012	PCA	FRE	C	30 °C	6000	700
17	11/09/2012	A	24/09/2012	PCA	FRE	C	30 °C	13500	650
18	11/09/2012	A	24/09/2012	PCA	FRE	C	30 °C	93500	0
19	17/09/2012	A	28/09/2012	PCA	OFRE	B	30 °C	171500	0
20	17/09/2012	A	28/09/2012	PCA	OFRE	B	30 °C	53500	0

21	17/09/2012	A	28/09/2012	PCA	OFRE	B	30 °C	43500	0
22	24/09/2012	A	07/10/2012	PCA	FRE	F	30 °C	2845000	21000
23	24/09/2012	A	07/10/2012	PCA	FRE	F	30 °C	3162333	8350
24	24/09/2012	A	07/10/2012	PCA	FRE	F	30 °C	2282666	966
25	24/09/2012	A	07/10/2012	PCA	FRE	F	30 °C	877333	550
26	24/09/2012	A	07/10/2012	PCA	FRE	F	30 °C	80333	400
27	24/09/2012	A	07/10/2012	PCA	FRE	F	30 °C	83666	1300
28	02/10/2012	A	18/10/2012	PCA	ORFE	A	30 °C	225666	0
29	02/10/2012	A	18/10/2012	PCA	ORFE	A	30 °C	944666	38000
30	02/10/2012	A	18/10/2012	PCA	ORFE	A	30 °C	470000	0
31	05/10/2012	A	24/10/2012	PCA	FRE	A	30 °C	49500	0
32	05/10/2012	A	24/10/2012	PCA	FRE	A	30 °C	101333	0
33	05/10/2012	A	24/10/2012	PCA	FRE	A	30 °C	2643333	0
34	08/10/2012	A	27/10/2012	PCA	CE	F	30 °C	156500	0
35	08/10/2012	A	27/10/2012	PCA	CE	F	30 °C	134500	0
36	08/10/2012	A	27/10/2012	PCA	CE	F	30 °C	328666	0
37	10/10/2012	A	30/10/2012	PCA	ORFE	G	30 °C	79000	0
38	10/10/2012	A	30/10/2012	PCA	ORFE	G	30 °C	214666	0
39	10/10/2012	A	30/10/2012	PCA	ORFE	G	30 °C	18000	0
40	15/10/2012	A	02/11/2012	PCA	CE	H	30 °C	24500	0
41	15/10/2012	A	02/11/2012	PCA	CE	H	30 °C	3000	0
42	15/10/2012	A	02/11/2012	PCA	CE	H	30 °C	0	0
43	18/10/2012	A	06/11/2012	PCA	FRE	I	30 °C	0	0

44	18/10/2012	A	06/11/2012	PCA	FRE	I	30 °C	21500	0
45	18/10/2012	A	06/11/2012	PCA	FRE	I	30 °C	9000	0
46	22/10/2012	A	12/11/2012	PCA	CE	J	30 °C	23500	0
47	22/10/2012	A	12/11/2012	PCA	CE	J	30 °C	3000	0
48	22/10/2012	A	12/11/2012	PCA	CE	J	30 °C	3000	0
49	24/10/2012	A	18/11/2012	PCA	CE	D	30 °C	1000	0
50	24/10/2012	A	18/11/2012	PCA	CE	D	30 °C	0	0
51	24/10/2012	A	18/11/2012	PCA	CE	D	30 °C	4000	0
52	26/10/2012	A	20/11/2012	PCA	FRE	K	30 °C	213000	0
53	26/10/2012	A	20/11/2012	PCA	FRE	K	30 °C	137200	0
54	26/10/2012	A	20/11/2012	PCA	FRE	K	30 °C	96500	0
55	12/11/2012	A	29/11/2012	PCA	FRE	F	30 °C	2000	0
56	12/11/2012	A	29/11/2012	PCA	FRE	F	30 °C	879333	100
57	12/11/2012	A	29/11/2012	PCA	FRE	F	30 °C	12000	0
58	10/01/2013	A	18/01/2013	PCA	ORFE	H	30 °C	17000	0
59	10/01/2013	A	18/01/2013	PCA	ORFE	H	30 °C	17000	0
60	10/01/2013	A	18/01/2013	PCA	ORFE	H	30 °C	0	0
61	14/01/2013	A	23/01/2013	PCA	FRE	F	30 °C	2783333	0
62	14/01/2013	A	23/01/2013	PCA	FRE	F	30 °C	141333	0
63	14/01/2013	A	23/01/2013	PCA	FRE	F	30 °C	4000	0
64	21/01/2013	A	30/01/2013	PCA	ORFE	I	30 °C	73500	0
65	21/01/2013	A	30/01/2013	PCA	ORFE	I	30 °C	466000	0
66	21/01/2013	A	30/01/2013	PCA	ORFE	I	30 °C	96000	0

67	21/01/2013	A	12/02/2013	PCA	CE	L	30 °C	12000	0
68	21/01/2013	A	12/02/2013	PCA	CE	L	30 °C	297000	0
69	21/01/2013	A	12/02/2013	PCA	CE	L	30 °C	295000	0
70	06/02/2013	A	21/02/2013	PCA	CE	J	30 °C	9500	0
71	06/02/2013	A	21/02/2013	PCA	CE	J	30 °C	173667	0
72	06/02/2013	A	21/02/2013	PCA	CE	J	30 °C	64333	20
73	11/02/2013	A	24/02/2013	PCA	CE	L	30 °C	79000	1893
74	11/02/2013	A	24/02/2013	PCA	CE	L	30 °C	82666	2826
75	11/02/2013	A	24/02/2013	PCA	CE	L	30 °C	47000	1946
76	13/02/2013	A	28/02/2013	PCA	CE	A	30 °C	6000	0
77	13/02/2013	A	28/02/2013	PCA	CE	A	30 °C	281000	40
78	13/02/2013	A	28/02/2013	PCA	CE	A	30 °C	36000	0
79	25/02/2013	A	14/03/2013	PCA	FRE	F	30 °C	474000	0
80	25/02/2013	A	14/03/2013	PCA	FRE	F	30 °C	857000	0
81	25/02/2013	A	14/03/2013	PCA	FRE	F	30 °C	0	0
82	27/02/2013	A	19/03/2013	PCA	CE	F	30 °C	700	0
83	27/02/2013	A	19/03/2013	PCA	CE	F	30 °C	800	0
84	27/02/2013	A	19/03/2013	PCA	CE	F	30 °C	12700	102
85	05/03/2013	A	27/03/2013	PCA	ORFE	M	30 °C	1200	0
86	05/03/2013	A	27/03/2013	PCA	ORFE	M	30 °C	2000	0

87	05/03/2013	A	27/03/2013	PCA	ORFE	M	30 °C	2900	0
88	05/03/2013	A	27/03/2013	PCA	ORFE	M	30 °C	1500	0

[a] Egg sources: A, Morrison saver; B, Sopa; C, True Scottish; D, Big and Fresh; E, Egg for soldier; F, Farmlay; G, M and S; H, One good egg; I, Morrison organic; J, Clenrath scottish egg; K, Scottish egg; L, Tesco value; M, Tesco organic.

Appendix II: Composition of Solutions and Reagents

L-Broth

Tryptone	5 g
Yeast extract	2.5 g
NaCl	2.5 g
Distilled H_2O	500 ml

L-Agar

Tryptone	5 g
Yeast extract	2.5 g
NaCl	2.5 g
Agar (1.5 %)	7.5 g
Distilled H_2O	500 ml

Ampicillin 20mg/ml

Ampicillin	20 mg
Distilled H_2O	10 ml

X-gal 40mg/ml

X-gal	40 mg
DMSO	10 ml

Glycerol 80%

Glycerol	80 ml
Distilled H_2O	20 ml

10x PBS (1L)

NaCl	80 g
KCl	2 g
Na2HPO4	7.62 g
KH2PO4	0.77 g
Distilled H_2O	800 ml

6x Gel loading buffer

Bromophenol blue	0.025 g
Xylene cyanol FF	0.025 g
Glycerol	3 ml
Distilled H_2O	10 ml

TE buffer

Tris-Cl	10 mM
EDTA	1 mM
pH	7.5

TAE buffer (1x)

Tris acetate	40 mM
Glacial acetic acid	20 mM
EDTA	1 mM
pH	8.0

50×TAE buffer

Tris Base	242 g
Glacial Acetic Acid	57.1 ml
EDTA	0.5 M
Distilled H_2O	up to 1000 ml

Appendix 3: Sequences of selected cloned isolates

>CL 1
CCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATT
GCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGT
TCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGTATCGTTGCCTTGGTA
AGCCGTTACCTTACCAACTAGCTAATACGGCGCGGGTCCATCTATAAGTGAT
AGCAAAACCATCTTTTACTTTAGAACCATGCGGTTCCAAATGTTATCCGGCA
TTAGCTCCGGTTTCCCGAAGTTATTCCAGTCTTATAGGTAGGTTACCCACGT
GTTACTCACCCGTCCGCCGCTAACTTCAAAGGAGCAAGCTCCTTATCCGTTC
GCTCGAC

>CL3
CTGATTAGGTACCGTCAGATGTGCACAGTTACTTACACATTTGTTCTTCCCT
AATAACAGAGTTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTC
CGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGA
GTCTGGACCGTGTCTCAGTTCCAGTGTGGCCGATCACCCTCTCAGGTCGGCT
ACGTATCGTTGCCTTGGTAAGCCGTTACCTTACCAACTAGCTAATACGGCGC
GGGTCCATCTATAAGTGATAGCAAAACCATCTTTTACTTTAGAACCATGCGG
TTCCAAATGTTATCCGGCATTAGCTCCGGTTTCCCGAAGTTATTCCAGTCTTA
TAGGTAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACTTCAAAGGAG
CAAGCTCCTTATCCGTTCGCTCGACTTGCAT

>CL5
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